# MICROBES IN THE ATMOSPHERE:

# PREVALENCE, SPECIES COMPOSITION, AND RELEVANCE TO CLOUD FORMATION

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All grown-ups were once children…
but only few of them remember it.
-The Little Prince

To my dad… because you believed I could reach the stars.
To my mom… you are my rock.
To my siblings… for show me what life is.
To my niece… I will build a better world for you. Lo prometo.

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| | |
|---|---|
| CCN | Cloud Condensation Nuclei |
| CPC | Condensation Particle Counter |
| d | Day |
| DMPS | Differential Mobility Particle Counter |
| DMS | Dimethyl Sulfide |
| $DMSO_2$ | Dimethyl Sulfone |
| gCCN | giant Cloud Condensation Nuclei |
| GRIP | Genesis and Rapid Intensification Processes |
| h | Hour |
| $H_2O_2$ | Hydrogen Peroxide |
| IN | Ice Nuclei |
| Ina | Ice nucleation protein/gene |
| km | kilometer |
| L | Liter |
| LB | Luria-Bertani |
| lpm | Liters per minute |
| m | meter |
| min | minute |
| mm | millimeter |
| NASA | National Aeronautics and Space Administration |
| nM | Nanomolar |
| $O_3$ | Ozone |
| OD | Optical Density |

| | |
|---|---|
| OH | Hydroxide |
| OPC | Optical Particle Counter |
| OTU | Operational Taxonomic Unit |
| PBAs | Primary Biological Aerosols |
| PBS | Phosphate-buffered saline |
| PCoA | Principal Coordinate Analysis |
| PCR | Polymerase Chain Reaction |
| qPCR | quantitative PCR |
| rpm | Revolutions per minute |
| rRNA | ribosomal ribonucleic acid |
| s | seconds |
| sp. | Species (singular) |
| spp. | Species (plural) |
| SSU | small subunit |
| U | units |
| UV | ultraviolet |
| wk | week |
| µl | microliter |
| µm | micrometer |

# SUMMARY

The composition and prevalence of microorganisms in the upper troposphere and their role in aerosol-cloud-precipitation interactions represent important, unresolved questions for biological and atmospheric sciences. Most studies to date were restricted to samples taken near the Earth's surface and/or to laboratory incubations that do not simulate well in-situ conditions. Further, the ability of most microbial taxa to serve (or not) as cloud condensation nuclei (CCN) remains uncharacterized. Therefore, the major objectives of this research effort were to characterize the composition and relative abundance of airborne bacteria in the troposphere, and measure their CCN activity under different growth conditions. To this end, low- and high-altitude air masses were sampled before, during, and after two tropical storms, and the microorganisms present in the samples were assessed based on quantitative PCR and microscopy. Viable bacterial cells represented on average around 20% of the total particles in the 0.25- to 1-μm diameter range, revealing that bacteria represent an important and underestimated fraction of coarse mode aerosols. Twenty bacterial isolates were recovered from these and additional rain samples, and the degree of their cell hygroscopicity was measured based on the contact angle of the bacterial cells with water. A wide range of contact angles was observed, with isolates ranging from very hydrophilic to very hydrophobic. The CCN activity of each isolate was studied by introducing aerosolized bacteria into a continuous flow stream-wise thermal gradient CCN counter. Hydrophilic bacteria were found to have a critical

supersaturation of 0.1% compared to hydrophobic bacteria, which showed a critical supersaturation of 0.2% or higher. These supersaturation conditions are relevant for cloud formation in continental areas. Collectively, these findings suggested that airborne bacterial cells represent an underappreciated aspect of the troposphere, with potentially significant impacts on the hydrological cycle, clouds, and climate.

# CHAPTER 1

# INTRODUCTION

## 1.1 Thesis rationale

The atmosphere is known to harbor many species of bacteria across all altitudes. The abundance and composition of airborne bacterial cells suggested that they could have an important and under-appreciated role in atmospheric chemistry and cloud formation. However, most studies to date have focused on sampling the boundary layer, leaving the upper troposphere poorly characterized. Yet, the upper troposphere is critical for the climate, e.g., cirrus (icy) cloud formation occurs primarily there. In order to provide a more comprehensive understanding of the role of bacteria in the atmosphere, we collected samples in the upper troposphere on board a specialized DC8 aircraft operated by the National Aeronautics and Space Administration (NASA), in addition to rainwater and near-surface air samples. We coupled metagenomic analysis with laboratory measurements of isolates recovered from these atmospheric samples to assess the cloud condensation nuclei activity of intact cells and their abundance and dynamics in the atmosphere.

## 1.2 Goals and research objectives

A few previous studies have suggested that biological particles in the atmosphere may be both abundant enough, and have the potential to form ice nuclei (IN) and cloud

condensation nuclei (CCN) to have an impact on regional climate (Möhler et al. 2007, Hoose, Kristjánsson, and Burrows 2010, Sesartic, Lohmann, and Storelvmo 2012). However, a quantitative assessment of the role of bacterial particles in cloud formation is still missing. The goal for this thesis research was to better appreciate the impact of biological particles in the atmosphere and provide quantitative data that can be incorporated into existing models of cloud formation and climate. To this end, the following objectives were addressed:

**1.2.1 Objective 1:** Characterize the taxonomic composition and abundance dynamics of airborne microbes by sequencing 16S rDNA gene amplicons in samples from different locations, altitudes and time points, and understand the habitat of origin on Earth of these taxa by comparing the resulting sequence against representative sequence from different habitats.

**1.2.2 Objective 2:** Understand the relationship between cloud condensation nuclei (CCN) activity and membrane hygroscopicity of bacterial cells isolated from atmospheric air and rain samples and quantify CCN activity of different taxa.

## 1.3 Literature review

### 1.3.1 Presence of bacteria in the atmosphere

When Louis Pasteur started working in proving wrong the spontaneous generation theory, he opened the doors to a new field of research: the aerobiology. In 1860, Pasteur started to study the microbes present in the atmosphere using the microscope (Pasteur 1860). He was the first scientist to do experimental aerobiology, collecting air samples from the streets of Paris to the high mountains in the Alps. Among his hypotheses, he stated that the higher you move in the atmosphere, the most sterile the air is, suggesting a decrease in concentration of microorganisms at high altitudes. In the 1900s, and with the introduction of airplanes to the field, new studies appear where samples were collected at higher altitudes, on board of planes. In 1935, Meier in collaboration with Charles A. Lindbergh, the American pioneer aviator, collected the first air samples over ocean and ice caps (Meier and Lindbergh 1935). Since then and with new technologies, air samples have been collected at different regions and altitudes across the globe, including at 20 km (in the stratosphere) (Amato et al. 2005, Amato, Parazols, et al. 2007b, Amato, Parazols, Sancelme, Mailhot, et al. 2007, Bowers et al. 2013, Bowers et al. 2009, Bowers, McLetchie, et al. 2011, Bowers, Sullivan, et al. 2011, Brodie et al. 2007, DeLeon-Rodriguez et al. 2013, Griffin 2008, Harrison et al. 2005, Lighthart et al. 2009, Meier and Lindbergh 1935, Proctor 1934, 1935, Wolf 1943). Microorganisms have been found in atmospheric air, cloud water, snow, and hailstones at concentrations of hundreds to

thousands of microorganisms in a cubic meter of air (Bowers et al. 2009, Brodie et al. 2007, DeLeon-Rodriguez et al. 2013, Harrison et al. 2005).

Scientists focused their efforts to study the composition and concentration of microbial species that are present in the atmosphere using culture-based methods. Among the most common organisms isolated from atmospheric samples were *Bacillus* spp. and *Pseudomonas* spp. (Amato, Parazols, et al. 2007b, Amato, Parazols, Sancelme, Mailhot, et al. 2007, Harrison et al. 2005). It is now known that such culture-based approaches can typically recover only about 1% of the total bacteria in a given sample, with this percentage ranging from 0.01% to 75%, the latter typically only in engineered bioreactors and some human samples (Chi and Li 2007, Heidelberg et al. 1997, Lighthart 2000). In the case of airborne bacteria, studies have shown that the aerosolization process reduces the culturability of gram-negative cells, leaving a large fraction of the community inaccessible to culture- based approaches (Heidelberg et al. 1997). Thus, the great majority of airborne bacteria, presumably between 90 and 99% of the total, remained inaccessible in these previous culture-based studies.

One way to advance the understanding of the role of the many bacterial cells in the atmosphere and to identify novel mechanisms important for their contribution to atmospheric processes (e.g., serving as cloud seeds), is through the use of culture-independent technologies such as metagenomics. In metagenomics, the power of genomic tools is applied to entire microbial communities, *in-situ*, bypassing the need to isolate and culture community members (Handelsman et al. 2007). Thus, metagenomics

provides novel insights into the diversity and function of microbial communities and the adaptations of the community to environmental conditions. The microbial communities present in the atmosphere remain poorly characterized with genomic approaches to date, mostly due to difficulties associated with obtaining representative samples, the low biomass of the airborne communities (which imposes additional challenges in obtaining enough DNA and protein material for analysis), and the traditional focus of large genomic surveys to the oceans and the human microbiome. A few metagenomic surveys of air samples have recently appeared, which were typically restricted to samples collected near the Earth surface (e.g., high mountains) and/or the analysis of a single gene, the small subunit ribosomal RNA or 16S rRNA gene (Bowers et al. 2013, Bowers et al. 2009, Bowers, McLetchie, et al. 2011, Bowers, Sullivan, et al. 2011, Brodie et al. 2007). The 16S rRNA gene serves as the best phylogenetic marker to identify the species found in a sample; and, by sequencing thousands of gene copies from a sample (as is often done with current methods), it can also provide reliable estimates of relative *in-situ* abundance (Hugenholtz, Goebel, and Pace 1998). However, the analysis of 16S rRNA genes cannot assess whether or not the corresponding species encode proteins important for cloud formation or survival in this environment and does not typically provide species-level resolution (Cole et al. 2010). Despite the limitations, recent studies based on the 16S rRNA gene have revealed that a much more diverse microbial community is present in the atmosphere compared to what was anticipated previously based on the results of culture-based efforts, thereby confirming the biases of the cultivation methods mentioned above (Bauer et al. 2002, Bowers et al. 2009, Bowers, McLetchie, et al. 2011, Bowers, Sullivan, et al. 2011, Brodie et al. 2007, Després et al. 2007). For example,

Bowers and colleagues studied the microbial communities in near surface air in the area of the Midwest of the United States and on top of Storm Peak in Colorado (Bowers et al. 2009, Bowers, McLetchie, et al. 2011, Bowers, Sullivan, et al. 2011). These studies revealed very diverse communities in the atmosphere, and also identified the habitats on Earth where these organisms originated. For instance, the microbial communities associated to air masses in these urban areas are related to those in dog feces (Bowers, Sullivan, et al. 2011).

Most research until now have shown the composition and concentration of bacterial communities in the atmosphere, however, little is know about what these organisms are doing while aloft. Anne-Marie Delort's group, at the Institute of Research of Clermort-Ferrand in France, has performed extensive studies looking at the contribution of airborne bacteria to atmospheric chemistry using microorganisms isolated from cloud water on the Puy du Dôme in France (Amato et al. 2005, Amato, Parazols, et al. 2007a, Vaïtilingom et al. 2013). These studies have found that microbial communities present in cloud water are capable of utilizing compounds that are present in clouds at temperatures as low at 10°C. However, these studies did not look at the composition of the microbial communities to elucidate the organisms responsible for the metabolic activities reported. This problem could be overcome with the integration of metagenomics analysis with functional experimental like those performed by Delort and colleagues.

Recently, the first metagenomic survey of atmospheric samples from near surface air and indoor air in New York City was published by Venter and colleagues (Yooseph et

al. 2013). Taxonomical analysis of metagenomic data showed significant differences between the indoor and outdoor air. Most notably, genera that are known to be part of the human microbiota were mostly present in indoor samples. In contrast to taxonomic composition, functional characterization did not differentiate between indoor and outdoor samples. Functional redundancy, despite taxonomical variation, has been observed in other environments, for example between marine and freshwater systems (Oh et al. 2011). However, no direct information on microbial activity in cloud chemistry and formation was provided in the previous study (Yooseph et al. 2013). Further, the low biomass collected in the Yooseph et al. (2013) study required the amplification of the total DNA present in these samples to obtain enough material for sequencing. However, the amplification process is known to introduce biases that can affect quantitative analysis and conclusions. Newer sequencing technologies can overcome this limitation by using protocols and chemistries that are able to sequence nanograms of input DNA efficiently (Parkinson et al. 2012, Adey et al. 2010).

In order to provide quantitative data on the influence of bacterial cells in cloud formation and atmospheric chemistry there is a need to characterize the CCN activity of many different taxa and assess the relative abundance of the taxa throughout the atmosphere. To this end, more extensive research and sampling are necessary compared to what has been accomplished to date, capturing several continental and marine zones that are representative of the atmosphere. Integrating the resulting data may provide the appropriate dataset to begin to answer important questions such as: what are microbes doing while present in the atmosphere? how do microbes survive there? and/or can

microbes influence formation of clouds?

### 1.3.2 Role of bacteria as cloud seeds

The formation of clouds depends on the condensation of water vapor on particles present in the atmosphere (water droplet formation) or the formation of ice crystals seeded by the particle when ambient temperature is below freezing point (Pruppacher, Klett, and Wang 1998). Accordingly, clouds can be divided in three main categories: warm clouds (i.e., clouds of liquid water droplets), cold clouds (i.e., clouds of ice crystals), and mixed-phase clouds (i.e., mixture of both liquid droplets and ice crystals). The formation of warm clouds required the presence of CCN and atmospheric conditions of supersaturated relative humidity (i.e., relative humidity over 100%; expressed as percentage over 100% relative humidity), this process is explained by the Köhler theory. The theory combines two physical properties of a particle, its size (Kelvin effect) and solubility (Raoult's Law), to explain the ability of a particle to serve as a nucleus for condensation [See p. 787-788 from Seinfeld and Pandis (2012)]. The Kelvin effect explains that small spherical particles required higher supersaturation conditions in order to serve as CCN than large particles, since larger particles provide more surfaces for water to condense. However, most particles in the atmosphere are small soluble particles such as salt. In that case, Raoult's Law explains that high concentration of solutes in a small droplet required smaller supersaturation conditions (See Chapter 3). Therefore, soluble particles tend to need lower critical supersaturation than small non-soluble

particles. The critical supersaturation refers to the supersaturation needed in order for half of the particles in a sample to activate as a CCN.

Cold clouds are made of ice crystals and occur at high altitude in subzero temperatures. In the absence of any particle or surface, pure water freeze at -38° C (i.e., homogeneous freezing) (Pruppacher 1995), however, several particles have the ability to increase the temperature at which water freezes. These particles, known as ice nuclei (IN), are mostly non-soluble particles such as dust, spores, pollen, and bacteria. There are four different processes that can lead to ice crystal formation in the atmosphere: homogeneous freezing (no IN present), immersion freezing (IN inside a super cooled water droplet), contact freezing (IN collide against a super cooled water droplet), and deposition freezing (sublimation over a IN).

Previous studies have found that bacterial cells can serve as CCN (Bauer et al. 2003, Franc and DeMott 1998) and IN (Möhler et al. 2008, Vali et al. 1976). However, the previous studies have analyzed a small number of bacterial taxa, they have frequently provided inconsistent results (e.g., dramatically different CCN or IN activities for the same bacterial taxa), and, perhaps more importantly, have not advanced our understanding of the mechanisms by which bacteria could serve as nuclei for the formation of clouds, with the possible exception the production of a highly efficient IN protein, inaZ (Bauer et al. 2003, Christner 2010, Franc and DeMott 1998). Despite the increase in the number of aerobiology studies, most of them still focus on characterizing the composition of airborne microbial communities. Therefore, the contribution of

microbial cells to cloud formation and atmospheric chemistry, and how their CCN/IN activity changes depending on prevailing environmental condition and the physiology state of the cells (e.g. spore vs. vegetative cells and exponential grown vs. stationary phase cells) remain poorly studied. For these reasons, this thesis focused on understanding and quantifying the role of bacteria as clouds seeds.

1.3.2.1 Bacteria as cloud condensation nuclei

Bacterial cells are wettable non-soluble particles that should follow the Kelvin effect. Therefore, and based on the idea of a completely wettable and non-soluble particle, bacterial cells should have a critical supersaturation of approximately 0.3% (Fig 1.1). However, bacterial cells isolated from cloud water and air samples were found to be CCN active at supersaturations as low as 0.07% (Bauer et al. 2003), not following the Kelvin effect. Even though the mechanisms by which bacteria acts as nuclei for water condensation are not clear, it has been suggested that the composition of the cell wall plays a critical role in the process (Bauer et al. 2003, Franc and DeMott 1998). Despite the potential of bacteria to act as CCN and their high CCN efficiency, there have not been many studies measuring the CCN activity of different taxa (Bauer et al. 2003, Franc and DeMott 1998), even the most studied airborne species and inaZ producing, *Pseudomonas syringae*. One reason for this is related, at least in part, to the low bacterial concentrations in the atmosphere compared to abiotic particles, especially in altitudes where warm clouds are present.

Although estimates indicate that abiotic CCN outnumber biological CCN in the atmosphere, bacterial cells could enhance the giant CCN (gCCN) population, which tend to produce larger water droplets (Kuba and Takeda 1983, Möhler et al. 2007). When conditions are met, these large droplets can grow fast enough to form raindrops embryos and promote precipitation (Yin et al. 2000); a phenomenon typically more common on continental warm clouds than on maritime warm clouds (Kuba and Takeda 1983). In the case of mixed phase clouds, gCCN not only increase the number of large

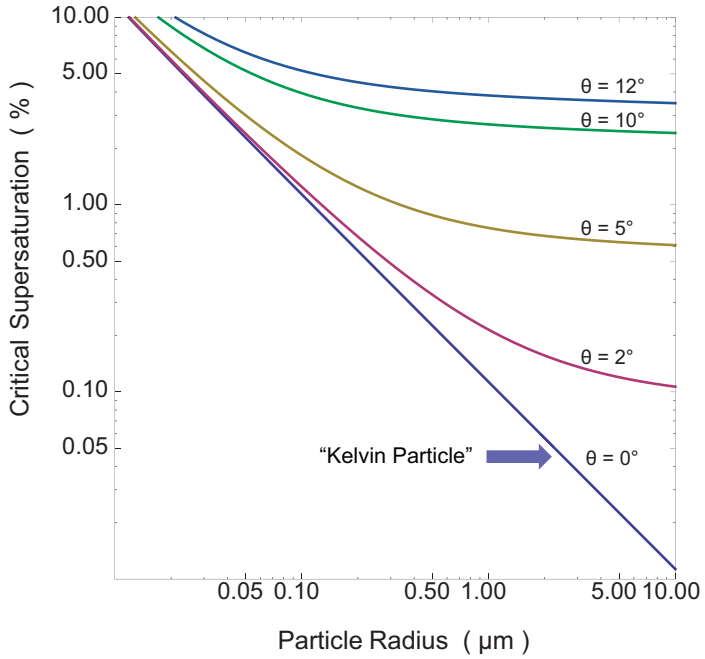


**Figure 1.1 Classical nucleation theory model.** Critical supersaturation (y-axis) for particles of different sizes (x-axis) and hygroscopicity levels. Blue line represents the expected critical supersaturations for completely wettable non-soluble particles (Kelvin particle). θ represents the contact angle value for each insoluble particle. Contact angle is measured to understand the interaction of water with the surface of an insoluble particle, by measuring the angle formed when water is in contact with the surface of the particle.

droplets, but also the collision rate between drops that accelerate precipitation (Yin et al. 2000). Therefore, even thought the concentration of bacteria in the atmosphere may represent a minor fraction of total CCN, these biological particles could have a significant

role in the formation of clouds and climate because they can contribute disproportionally high to the formation of gCCN.

A few studies have suggested that CCN activity could be linked to the membrane composition and hydrophobicity. For instance, McDonald (1964) calculate the theoretical critical supersaturation of insoluble particles that have small contact angles. For example, particles with water contact angle of over 12° are expected to require supersaturations higher than 5% (Fig 1.1). The contact angle is a value used to quantify the interaction between a liquid and a surface, by determining the angle formed between the two. For example, if the contact angle of a drop of water over a bacterial lawn has a small value means that such bacteria have higher affinity to water, meaning they are hydrophilic. Van der Mei and others, used this technique to measure the hygroscopicity of bacterial cells in relation to cavity research (Van der Mei, Bos, and Busscher 1998, Van der Mei et al. 2003, Van der Mei, Weerkamp, and Busscher 1987, Weiss et al. 1982). These studies found that the contact angle of bacterial cells can range between 16° to 106°, depending on the organism used in the study. None of these studies, however, related bacteria hygroscopicity with their CCN ability, since the focus was related to surface interaction, not measuring CCN activity. Yet, it is important to quantitatively evaluate how bacterial membrane hygroscopicity relates to CCN activity and directly test the theoretical predictions.

1.3.2.2 Bacterial ice nuclei

Homogeneous freezing of liquid water in the atmosphere is known to happen only at temperatures lower than -38°C, but in the presence of a particle with ability to initiate ice formation, this process can happen at higher temperatures. These particles are called ice nuclei (IN). Some of the best known IN are pollen and bacterial cells. In the 1970s, Vali (Vali et al. 1976) found that a plant pathogen, *Pseudomonas syringae,* could induce freezing at -2°C, due to an outer membrane protein, making this bacterium the most efficient known ice nuclei. Aerobiology studies have found this organism present in samples collected at different altitudes in the atmosphere and in different types of atmospheric samples (e.g., rain, snow, air, and hail) (Garcia et al. 2012, Hill et al. 2014, Šantl-Temkiv et al. 2013, Constantinidou et al. 1990, Monteil et al. 2012, Morris et al. 2008).

For many decades several research groups have tried to explain the presence of this organism in atmospheric samples. David Sands and colleagues (Sands et al. 1982) proposed the 'bioprecipitation feedback hypothesis', in which it is explained how bacteria such as *Pseudomonas syringae,* are lifted to the atmosphere when dry conditions prevail on Earth by movement of plant leaves and stomata, once in the atmosphere this organisms can promote the formation of ice crystals and induce precipitation, to colonize a more suitable environment. A recent study found an increase in biological aerosols and IN particles during rain events in forested areas (Huffman et al. 2013), which provides support to the bioprecipitation feedback hypothesis.

Bacteria that are active as ice nuclei are known to have an outer membrane protein encode by the *inaZ* gene (Vali et al. 1976). This protein is 1034-1567 residues long and has a circular loop of 16-residue motif that is repeated several times (Kumaki et al. 2008, Warren, Corotto, and Wolber 1986). This repetitive unit is believed to provide the activity, by serving as a template for the formation of ice crystals (Kawahara 2002). The inaZ protein has been found among several *Gammaproteobacteria* species, mostly plant pathogens (Lindow, Arny, and Upper 1978, Vali et al. 1976). The presence of this gene/protein in the atmosphere remains poorly understood. A recent study designed a set of primers that can be used to quantify the presence of this gene in the environment (Hill et al. 2014). However, the samples used only included precipitation and near surface air, and no quantification of the mid-to-upper troposphere was performed. Information regarding the presence of this gene at different altitudes would be important for better appreciating the role of bacteria in cloud formation and potentially, providing the necessary quantitative data for incorporation into cloud-resolving models.

### 1.4 Knowledge gaps

Bacteria present in the atmosphere can be passive or active, and it is important to note that even inactive, resting cells can potentially serve as CCN or IN. Despite recent work and the introduction of new technologies to aerobiology, however, it is still highly debatable whether or not airborne cells significantly influence cloud formation and atmospheric chemistry. All relevant studies performed to date, provide some evidence on

how bacterial cells can serve as CCN and IN; however, no quantitative data and direct, in-situ measurements have been collected for many taxa and environmental conditions. Also, the concentration and CCN/IN potential of bacteria in the upper troposphere remain poorly characterized.

To close some of the knowledge gaps in this area, this thesis is focused on three main research questions:

- What is the composition and concentration of bacterial cells at high altitudes? (**Chapter 2**)
- What is the level of CCN activity of different bacterial taxa and how is the activity affected by growth conditions? (**Chapter 3**)

To answer these questions, laboratory measurements of CCN activity coupled with culture-independent (a.k.a. metagenomics) analysis of atmospheric samples were employed. Metagenomic analysis of 16S rRNA gene amplicons was used to understand the composition and concentration of bacteria in the lower and upper atmosphere. A new instrumentation pipeline based on a continuous flow stream-wise thermal gradient CCN counter (Roberts and Nenes 2005) was used to measure CCN activity of several bacterial isolates obtained from different locations and altitudes.

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# CHAPTER 2

# THE MICROBIOME OF THE UPPER TROPOSPHERE: SPECIES COMPOSITION AND PREVALANCE, EFFECTS OF TROPICAL STORMS, AND ATMOSPHERIC IMPLICATIONS

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## 2.1 Abstract

The composition and prevalence of microorganisms in the middle-to-upper troposphere (8-15 km altitude) and their role in aerosol-cloud-precipitation interactions represent important, unresolved questions for biological and atmospheric science. In particular, airborne microorganisms above the oceans remain essentially uncharacterized, as most work to date is restricted to samples taken near the Earth's surface. Here we report on the microbiome of low- and high-altitude air masses sampled onboard the National Aeronautics and Space Administration DC-8 platform during the 2010 Genesis and Rapid Intensification Processes campaign in the Caribbean Sea. The samples were collected in cloudy and cloud-free air masses before, during, and after two major tropical hurricanes, Earl and Karl. Quantitative PCR and microscopy revealed that viable bacterial cells represented on average around 20% of the total particles in the 0.25- to 1-μm diameter range and were at least an order of magnitude more abundant than fungal cells, suggesting that bacteria represent an important and underestimated fraction of micrometer-sized atmospheric aerosols. The samples from the two hurricanes were

characterized by significantly different bacterial communities, revealing that hurricanes aerosolize a large amount of new cells. Nonetheless, 17 bacterial taxa, including taxa that are known to utilize C1-C4 carbon compounds present in the atmosphere, were found in all samples, indicating that these organisms possess traits that allow survival in the troposphere. The findings presented here suggest that the microbiome is a dynamic and underappreciated aspect of the upper troposphere with potentially important impacts on the hydrological cycle, clouds, and climate.

## 2.2. Introduction

Airborne microorganisms likely play an important role in cloud formation and precipitation for a number of reasons. First, the concentration of microbial cells (typically, 0.1-3 mm in diameter) in the lower troposphere (< 5 km altitude) is thought to be comparable to non-biological supermicron ice nuclei (IN) (Despres, Huffman et al. 2012). Second, several bacterial plant pathogens are known to promote heterogeneous freezing of ice through the action of an outer membrane protein (the inaZ protein), which serves as nucleation center (Vali, Christensen et al. 1976, Möhler, DeMott et al. 2007). The prime biological function of the protein is to damage the leaves of plants and facilitate plant infection by the pathogens. When suspended in the atmosphere, these organisms can act as efficient IN at temperatures as high as -2°C (Vali, Christensen et al. 1976), much higher than any non-biological IN. Third, several bacterial species are known to act as efficient cloud condensation nuclei (CCN) (Bauer, Giebl et al. 2003); because of their large size and concentration, they can contribute to the population of

"giant CCN" (Möhler, DeMott et al. 2007, Amato 2012). Giant CCN can promote the formation of precipitation by acting as collector drops that form drizzle.

However, a comprehensive understanding of the CCN and IN efficiency of different microbial types (e.g., bacteria vs. fungi) and species, as well as their spatial and temporal distribution in the troposphere, particularly at high altitudes, is essentially lacking. This severely limits our understanding of the importance of airborne microbial cells for cloud formation and the hydrological cycle (Christner 2012, Despres, Huffman et al. 2012). Furthermore, most studies to date on the atmospheric microbiome are restricted to samples collected near the Earth's surface (e.g., top of mountains). The tropospheric microbial communities at high altitudes and in air masses over marine/oceanic regions remain poorly characterized, mostly due to difficulties associated with obtaining representative samples of sufficient biomass (which imposes additional challenges to recover enough DNA and protein material for analysis). Little is known about the composition, spatial and temporal variability of these microbial communities, how they adapt to their environment, and if they are viable and can metabolize organic constituents present in the atmosphere. It is also important to understand how atmospheric processes that can aerosolize and/or precipitate microbial cells, such as major storm systems (tropical cyclones and hurricanes), affect microbial community composition and function. Advancing our understanding of these issues will also contribute to improved models of microbial disease dispersal and microbial biogeography (Martiny, Bohannan et al. 2006, Womack, Bohannan et al. 2010, Christner 2012).

A few culture-independent molecular studies, which analyzed atmospheric air or snow water collected on the ground. These studies have revealed considerably more diverse bacterial and fungal communities to be present in the atmosphere of several ecosystems such as the Amazon River and urban cities compared with what was previously anticipated on the basis of the results of culture-based efforts (Brodie, DeSantis et al. 2007, Després, Nowoisky et al. 2007, Bowers, Lauber et al. 2009, Poschl, Martin et al. 2010, Bowers, McLetchie et al. 2011, Bowers, Sullivan et al. 2011). Furthermore, microorganisms commonly found in soil (Lauber, Hamady et al. 2009) and vegetation (Andrews and Harris 2000), including plant pathogens with IN activity such as *Pseudomonas syringae*, were frequently observed in the previous culture-independent surveys. Although the relevance of the findings of these studies for the microbial communities of the middle-to-upper troposphere remains unknown, the identification of complex microbial communities warrants further investigations of the role of the diverse microbes found in the atmosphere and their potential to serve as CCN or IN.

Toward closing these knowledge gaps, we obtained several high-altitude samples at ~10 km above sea level during the National Aeronautics and Space Administration Genesis and Rapid Intensification Processes (GRIP) campaign, which was focused on tropical hurricanes that developed in the Caribbean Sea and the midwestern Atlantic Ocean in 2010. Here, we report on our initial efforts to characterize the microorganisms in these samples, including the analysis of sequenced amplicons of the small subunit ribosomal RNA gene (SSU rRNA), the gene that serves as the best phylogenetic marker to identify the species present in the samples and estimate their in-situ abundance (Hugenholtz, Goebel et al. 1998). Our results provide important insights into the species

composition, concentration, and dynamics of the microbial communities of the upper troposphere and the potential impact of airborne microbial cells on cloud formation.

## 2.3 Methods

**2.3.1 Sample collection.** Samples were obtained onboard the NASA Dryden Flight Research Center DC-8 aircraft platform deployed during the NASA GRIP campaign (see Table 2.1). Bioaerosols were collected on Whatman cellulose nitrate membranes of 0.22 μm pore size and 47 mm in diameter by filtering air from outside the aircraft using a vacuum pump. Filters were placed in four parallel lines to allow duplicates collection and a field/handling blank during each flight. Field blanks were placed in a line where no air was pumped through. Airflow was controlled using a critical flow orifice to a constant volumetric sample rate of about 20 L $min^{-1}$ per filter. All filters were stored at 4°C until processed.

**2.3.2 DNA extraction and bacterial and fungal cell quantification.** Membranes were incubated overnight at -80°C, prior to DNA extraction. A hot phenol:chloroform DNA extraction followed by DNA ethanol precipitation was used, as previously described (DeLong, Preston et al. 2006), with the only modification of a 5 min incubation at 65°C with the first phenol addition. To determine the SSU rRNA gene copy number, SYBR Green-based qPCR with universal primers was used. The qPCR reaction had a final concentration of 300 nM of each primer (Musher, Fredricks et al. 2004, Ritalahti, Amos et al. 2006), 1X of SYBR PCR mix (Applied Biosystems), and 2 μl of sample;

PCR amplification was performed as described (Ritalahti, Amos et al. 2006). Cell counts were performed using an Axion Observer D1 epifluorescence microscope (Zeiss). Bacterial cells were detached from the membranes by shaking each membrane in a PBS-buffer as described (Bowers, Lauber et al. 2009). Suspended cells were subsequently stained using the Invitrogen Live/Dead staining kit. Live to dead ratio was quantified for each of the samples based on the average ratio of green to red cells from sixteen 90x90 $\mu m^2$ fields per sample. Average cell numbers were expressed as concentration by scaling to the total area of the filter divided by the size of the wettable area of the filter and extrapolated to the total volume of air that was flown through the filter during sampling.

**2.3.3 SSU rRNA gene pyrosequencing and community composition analysis.**

SSU rRNA gene amplification was performed with barcoded-primers for the V1-V3 regions. PCR amplification reaction consisted of 500nM of each primer, 1X AccuPrime™ PCR Buffer II, and 1U of the AccuPrime™ Taq DNA Polymerase High Fidelity (Invitrogen). The PCR amplification program consisted of an initial denaturation step for 2 min at 95°C followed by 30 cycles at 95°C for 20 s, 56°C for 30 s and 72°C for 1:45 min. Barcoded-PCR fragments were cleaned with the Gel Purification kit from QIAGEN and quantified using picogreen. Each sampled was diluted to a final concentration of $10^9$ molecules $\mu l^{-1}$ and pooled together. The pooled sample was sequenced using the Roche 454 GS-FLX-Ti and the protocol provided by the manufacturer. Sequences were analyzed using the QIIME v.1.04 (Caporaso, Kuczynski et al. 2010). Briefly, sequences were first separated using the barcode assigned to each sample and then denoised and checked for chimeras using the "Denoise" and

"Chimera_Check" modules included in the QIIME package with default settings. Sequences were clustered into different OTUs, using a cut-off of 97% nucleotide sequence identity. The taxonomic affiliation of each OTU was determined using the GreenGenes database at the family and genus level. Phylogeny-based beta-diversity was estimated using the weighted UniFrac distance metric available in QIIME. PerMANOVA was performed using the vegan package in R and the Chao1 richness estimates based on the equations used in the Mothur software (Schloss, Westcott et al. 2009). SSU rRNA sequences were deposited to the GenBank (accession number SRA056067) and MG-RAST databases.

**2.3.4 Assignment to a habitat.** A subset of the GreenGenes database (DeSantis, Hugenholtz et al. 2006) was used to assign each OTU recovered to an habitat on Earth. To form the subset, full-length GreenGenes sequences with available information on the source of origin were selected. These sequences were subsequently classified, in-house, into four different categories: *Marine* (combining coastal and open ocean sequences; 11,447 sequences), *Freshwater* (5,099 sequences), *Terrestrial* (soil and plant-associated sequences combined; 3,799 sequences), and *Feces* (70,353 sequences) and grouped in clusters using a 97% sequences identity cut-off. Representative sequences of each OTU or raw sequences were assigned to a cluster, and hence to one of the four categories, based on a BLAT search and a >97% nucleotide sequence identity cut-off (Kent 2002). To normalize for the different representation of categories (habitats) in the GreenGenes database (e.g., soil are underrepresented relative to aquatic habitats), the analysis was

repeated by sampling at random 1,000 sequences from GreenGenes database for each habitat.

## 2.4 Results

**2.4.1 Samples collected and cell concentrations.** The GRIP campaign operated one flight off the coast of California, one intercontinental flight across the United States (transit flight from California to Florida), and seven flights in the areas of the Gulf of Mexico, the Caribbean Sea, and the midwestern Atlantic Ocean (Fig 2.1) during which samples were taken. The flights spanned a total period of about 6 wk (August 10, 2010 - September 20, 2010) and included flights during Hurricanes Earl and Karl. For each hurricane, samples were collected at the hurricane's intensification (category 1) phase and later (category 2 and 3) phases. Flights also included sampling the cloudy environment before Hurricane Earl (August 17, 2010), low altitudes (September 17, 2010, during the time of Hurricane Karl but sampling air masses undisturbed by the hurricane; 1-4 km vs. ~10 km in all other flights), and a cloud-free air mass (September 20, 2010) after the passing of Hurricane Karl. During each flight, biomass from an average of 6 $m^3$ of ambient air was collected per sample, using two separate sampling lines (duplicate samples; Table 2.1). Each sample was collected over a period of 3 h, on average (ranging from 1 to 5 h); thus, the samples were presumably representative of the air masses sampled. In addition, several filters, used as handling blanks, were connected to a sampling line with no airflow.

Both molecular and microscopic approaches were employed to quantify the concentrations of bacterial and fungal cells in the samples. Quantitative PCR (qPCR) of the gene SSU rRNA revealed an average bacterial SSU rRNA gene copy number of $2.0x10^4$ copies $m^{-3}$ (ranging from $3.0x10^3$ to $9.0x10^4$ copies $m^{-3}$). Assuming that the average bacterial genome has about four rRNA copies based on 1,144 complete genomes available in the *rrnDB* database at the beginning of 2012 (Klappenbach, Saxman et al. 2001), our results revealed an average bacterial concentration of $5.1x10^3$ cells $m^{-3}$. Field blanks showed at least an order of magnitude lower SSU rRNA gene copy abundance compared with the SSU

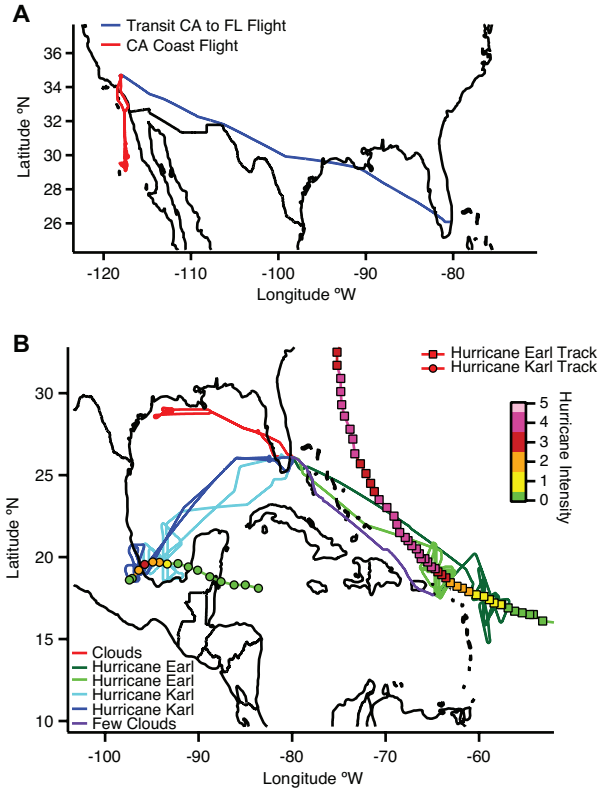


**Figure 2.1 Flight trajectory maps.** **(A)** Flights conducted in the west coast (red) and across the USA (blue). **(B)** Flights conducted in the area of the Caribbean Sea and the mid-western Atlantic Ocean. The route of each flight is color-coded (see figure key). The trajectory of Hurricane Earl (n) and Karl (□) are colored-coded based on the intensity of the hurricane at each time point (scale bar).

rRNA genes in the corresponding field samples or, in about half of the cases, no detectable SSU rRNA genes (Fig. 2.2 *A* and *B*), suggesting that the field samples have microbial cells that truly represent the tropospheric microbial communities. [Note that the weak, positive qPCR signal observed in a few of the blanks is most likely due to our approach to sterilize the filters and filter holders, which involved autoclaving at 120° C for 30 min. Thus, it is possible that DNA from dead (autoclaved) cells remained on the filters and provided a signal during qPCR. Consistent with these interpretations, we failed to obtain enough DNA for SSU rRNA amplicon pyrosequencing from any of the blanks (see below). Also note that more starting DNA material is required for amplicon sequencing compared to qPCR, which presumably accounts, in part, for the results obtained].

Fungal cells were also present in the samples based on qPCR analysis, at about an order of magnitude lower concentration than bacterial cells, with an average concentration of $6.8x10^2$ copies $m^{-3}$ (ranging from $1.9x10^2$ to $1.3x10^3$ copies $m^{-3}$). Given also that fungal genomes typically encode a higher number of rRNA copies than bacteria (ranging form 30 - 100 copies per genome, depending on the species considered), these results suggest that bacteria are at least two orders of magnitude more abundant relative to the fungi at high altitudes. Particles of similar size to that of bacterial cells (0.1 – 3 mm) tend to have greater residence times in the atmosphere compared with larger particles (such as fungal cells and spores that are typically > 3 μm in diameter), which probably accounts, at least in part, for the differences in cell concentrations observed

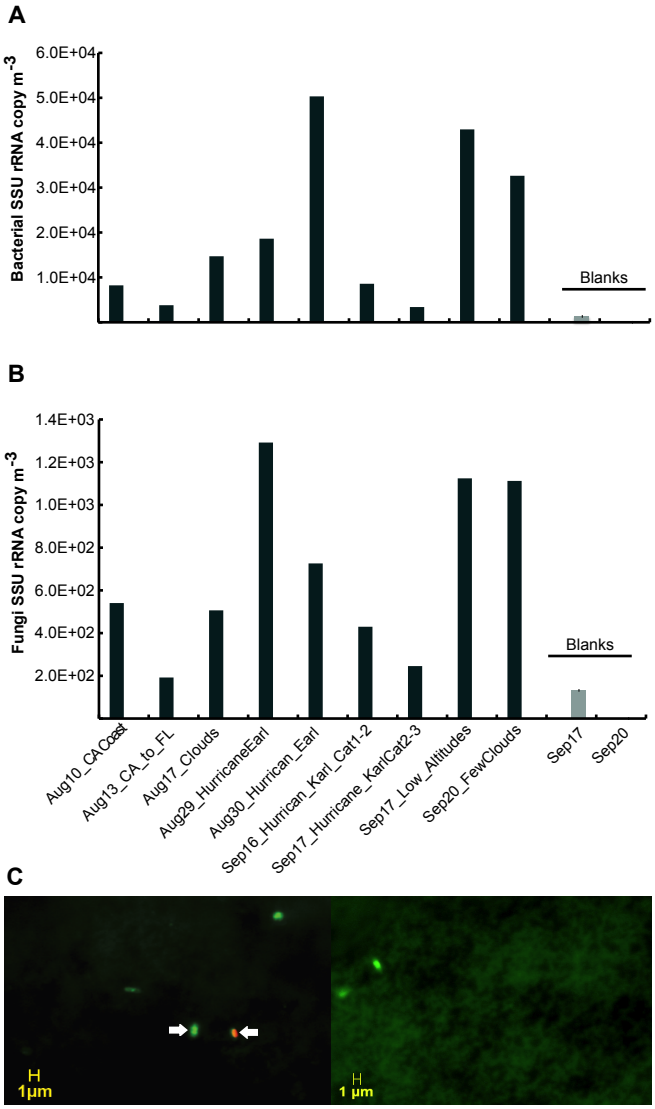


**Figure 2.2 Quantification of bacterial and fungal cells in samples from high altitudes in the atmosphere.** Concentration of bacterial ***(A)*** and fungal ***(B)*** cells based on qPCR analysis of SSU rRNA gene copies in the samples. Note that samples are ordered by the collection time on the x axis except for the blank samples, which are shown at the rightmost part of the graphs in light gray. ***(C)*** Live/dead microscopy image of two samples from the California coast and transit flights. Green-stained cells represent cells with viable/intact membrane (e.g., cell indicated by left arrow), and re/yellow-stained cells represent cells with a damage membrane (e.g., cell indicated by right arrow).

. Bacterial and fungal cells had similar concentration patterns across all samples analyzed with the exception of the samples taken during Hurricane Earl, where bacterial

cells increased and fungi cells decreased in samples taken over 2 consecutive days (Fig. 2.2 *A* and *B*).

Microscopy-based cell counting for the same samples revealed an average concentration of $1.5x10^5$ cells $m^{-3}$ (Table 2.1). The higher cell concentration based on microscopy relative to qPCR by about one order of magnitude is not unexpected and is likely attributable to a smaller average SSU rRNA copy number of the organisms in the samples relative to the copy number used in the calculations and/or qPCR underestimating cell abundance due to technical limitations. The technical limitations include primer mismatches, not all cells in the sample being lysed for DNA extraction (e.g., spores can be resistant to lysis), and DNA loss during extraction. Microscopy-based estimates might also represent overestimates due, for example, to abiotic particles being stained and counted (erroneously) as cells. However, the limitations of microscopy should be comparatively less important for the results obtained. The microscopy observations also revealed that the fraction of viable cells in the samples ranged from 60 to almost 100% of the total cells based on the live/dead cell viability test (Table 2.1 and Fig. 2.2 *C*), suggesting that these organisms can survive the adverse conditions present in the atmosphere. Samples taken during hurricanes had typically higher numbers of total and viable cells compared with samples taken off the coast of California and during the transit flight (Table 2.1 and Fig. 2.2 *A*), revealing that hurricanes aerosolize a large amount of new cells and these cells remain viable for at least a few days (because our samples taken around a hurricane event spanned a period of about 1 wk).

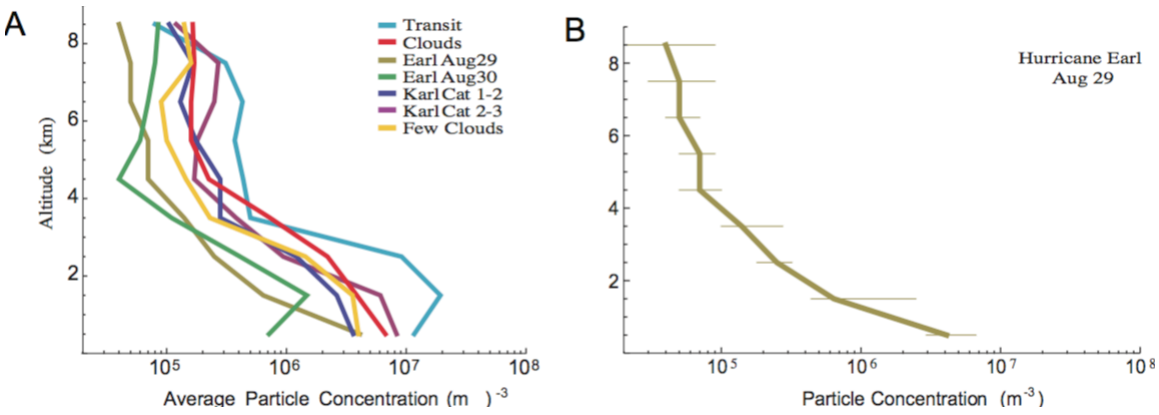


**Figure 2.3 Average concentration of particles (A) and an example of concentration variability for one flight (B).** Concentrations of total particles ranging from 0.5 to 10μm in diameter were measured using an Aerodynamic Particle Sizer (APS). APS can reliably retrieve particle concentrations up to 8 km in altitude. The error bars represent the variation (inter-quartile distance) of all the measurements taken every 0.5 km for the first Hurricane Earl flight.

The microscopy analysis also revealed that most bacterial cells in a sample (typically, >80% of the total) were 0.25 - 1 μm in diameter (Table 2.2 and Fig. 2.2 *C*). Total particle concentration in the 0.25 to 1 μm diameter range, including biological and abiotic particles, was measured during each flight (in real time) using an Ultra-High Sensitivity Aerosol Spectrometer (Droplet Measurement Technologies). As expected, particle concentration decreased with altitude from an average of $5.9 \times 10^6$ particles $m^{-3}$ at 0-1 km to $2.6 \times 10^5$ particles $m^{-3}$ at 7-8 km (Table 2.1). These results were also consistent with independent measurements obtained using an aerodynamic particle sizer (TSI Inc., model 3321; Fig. 2.3). Based on the microscopy cell counts, bacterial cells constituted a substantial fraction of the total coarse-mode particles at high altitudes (8-10 km), ranging from 3 to almost 100% in some of the hurricane samples [about 20% on average, (Table 2.1)]. Bacterial contribution was significant even based on the qPCR results, representing about 2% of the total particles, on average. These findings reveal that viable cells represent an important component of the micron- sized aerosols at high altitudes,

contrasting with near-surface aerosols that are typically characterized by a much lower cell fraction, on the order of 1 cell in a million of abiotic CCN particles (Hoose, Kristjánsson et al. 2010).

**2.4.2 Microbial community composition.** Bacterial SSU rRNA gene amplicon sequences were obtained using pyrosequencing technology (Roche 454) and were processed, trimmed, and denoised using the QIIME pipeline

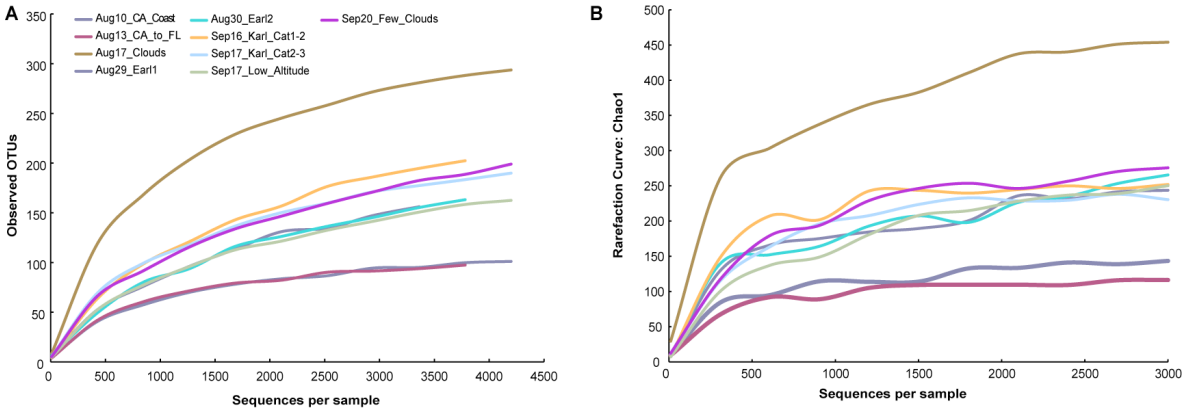


**Figure 2.4 Rarefraction curves of SSU rRNA gene amplicon sequences recovered from the GRIP samples.** Curves represent the number of unique OTUs recovered (vertical axis), defined at the 97% nucleotide sequence identity level, for the number of sequences analyzed (horizontal axis) and reflect the extent of OTU diversity within the samples and what fraction of this diversity was sampled. **(B)** Chao1 richness estimate rarefaction curve based on 3,000 sequences randomly drawn from each sample.

(Caporaso, Kuczynski et al. 2010), resulting in over 45,000 sequences across all samples (about 5,000 per sample, on average). A total of 314 operational taxonomic units (OTUs), defined at the 97% nucleotide sequence identity level, were identified among all sequences, ranging from 99 to 299 OTUs per sample. Notably, samples collected over the continental United States had lower species richness compared with the hurricane-related samples based on the Chao1 richness estimate, i.e., 129 and 113 for the California coast

and transit flights, respectively, vs. an average of 341 for the hurricane samples (147 and 118 vs. 251, respectively, when normalized for the number of sequences in each sample by randomly subsampling 3,000 sequences per sample). No other significant difference in species richness was observed. Rarefaction analysis revealed that the number of unique SSU rRNA gene diversity in most of the samples was close to being saturated by sequencing (Fig. 2.4), revealing that the tropospheric communities are less complex compared to many habitats on Earth such as soils [e.g., ref. (Delmont, Simonet et al. 2012)]. The vast majority of the OTUs were classified *Alpha-* and *Betaproteobacteria*. Based on the taxonomical classification of the SSU rRNA gene sequences recovered, *Afipia* sp. (*Alphaproteobacteria*) comprised over 50% of the total communities sampled off the California coast and during the transit flights. This group was reduced to less than 20% of the community and apparently replaced by members of the *Burkholderiales* order (*Betaproteobacteria*) as an effect of Hurricane Karl (the possibility that the high relative abundance of these groups is due to amplification biases is discussed below). The abundances of all OTUs in each sample are provided in Appendix A.

Samples related in time or space (same geographic region) tended to show more similar community composition patterns compared with unrelated samples (PerMANOVA, $P < 0.05$). For example, the samples taken off the coast of California and during the transit flight, as well as the four samples taken during and 3 d after Hurricane Karl, showed almost indistinguishable community compositions (Fig. 2.5 *A*). However, the communities at the aftermath of the two hurricanes were dramatically different compared with those before the beginning of the hurricanes. However, the

communities at the aftermath of the two hurricanes were dramatically different compared with those before the beginning of the hurricanes. These results suggest that hurricanes have a major impact on the composition of the tropospheric communities based apparently on the large number of new microbial cells they aerosolize and the precipitation scavenging of preexisting cells. Note, for example, the absence of *Ralstonia* during Earl compared with the high abundance of this group during Hurricane Karl in Fig. 2.5. Consistent with these interpretations, principal coordinate analysis of SSU rRNA gene sequences revealed three major clusters among the GRIP samples: two clusters representing the samples taken during each of the hurricanes and one cluster representing the samples taken off the California coast (Fig. 2.6, PerMANOVA, $P < 0.05$). Nonetheless, additional samples need to be analyzed before more quantitative conclusions about the effect of tropical hurricanes on tropospheric microbial communities can emerge.

A set of 17 OTUs was present across all samples, albeit in varied abundances, indicating that these organisms represent core members of the microbiome of the middle and upper troposphere. Given that the samples analyzed represent geographically distant locations (e.g., California vs. Caribbean) and cloud-free, cloudy, and tropical storm environments, these OTUs represent organisms that apparently possess traits to survive long periods of time at high altitudes in the atmosphere. Two of the core families were the *Methylobacteriaceae* and *Oxalobacteraceae*, members of which metabolize oxalic acid, a main product of cloud-mediated chemistry and one of the most abundant dicarboxylic acids in the atmosphere (Kawamura and Usukura 1993, Falkovich, Graber et al. 2005). Hence, it is conceivable that these groups could remain metabolically active in

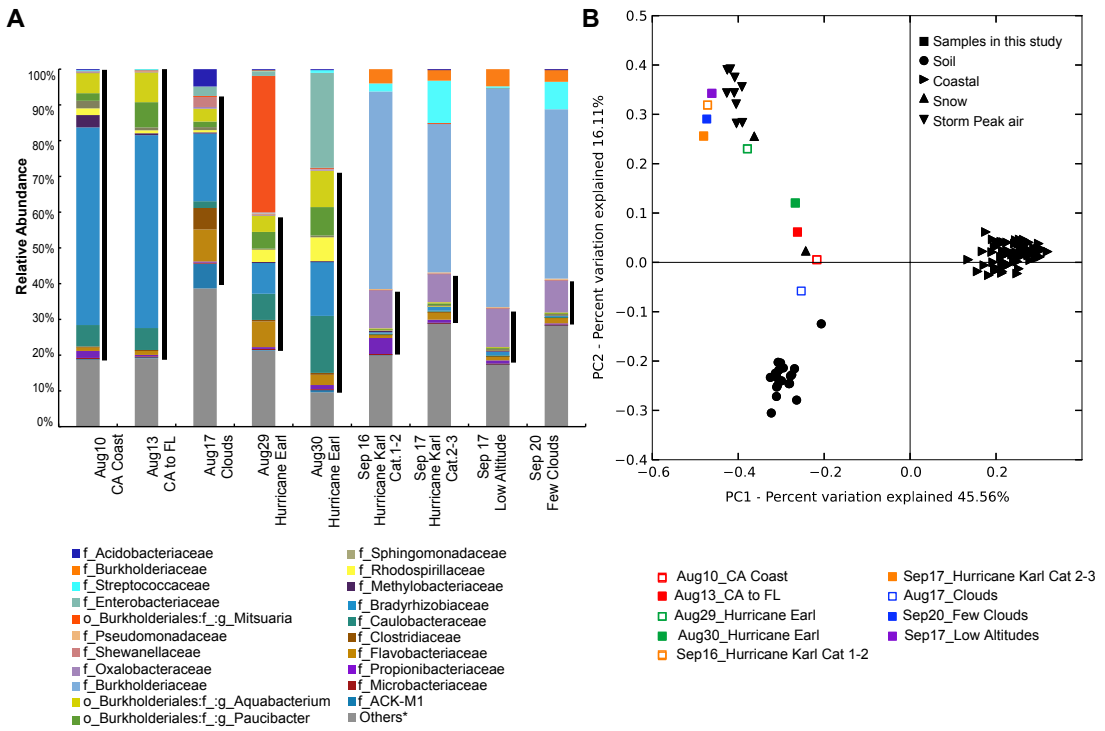


**Figure 2.5 Composition of tropospheric bacterial communities.** ***(A)*** Relative abundance (y axis) of taxa (see key) represented by the partial SSU rRNA gene sequences recovered in each sample (x axis). Black vertical lines next to the bars underline the core OTUs that are present in all samples. "*Others," refer to *Appendix A*. ***(B)*** Principal coordinates analysis based on the β- diversity values calculates by the weighted UniFrac distance metric of samples collected during the GRIP campaign and samples from previous studies (see key). Samples from this study are represented with open and closed squares and color-coded as follows: red California/transit; blue, clouds; purple, low altitude; green, Hurricane Earl; orange, Hurricane Karl.

clouds, although their ability to use oxalic acid while present in the atmosphere awaits experimental verification. The relative abundance of the core OTUs varied with time and geographic location. For example, the core OTUs substantially decreased in abundance at the aftermath of Hurricane Earl but remain present, even at relatively low numbers, in the sample taken 3 d after the impact of Hurricane Karl. Microbial cells of 0.25 - 1μm in diameter (Fig. 2.2 *C*) take several days (to a few weeks) to gravitationally settle from high altitudes and the samples analyzed here spanned a period of more than a

month. Therefore, the core members are apparently able to stay aloft and remain viable (microscopic observations) for at least several days.

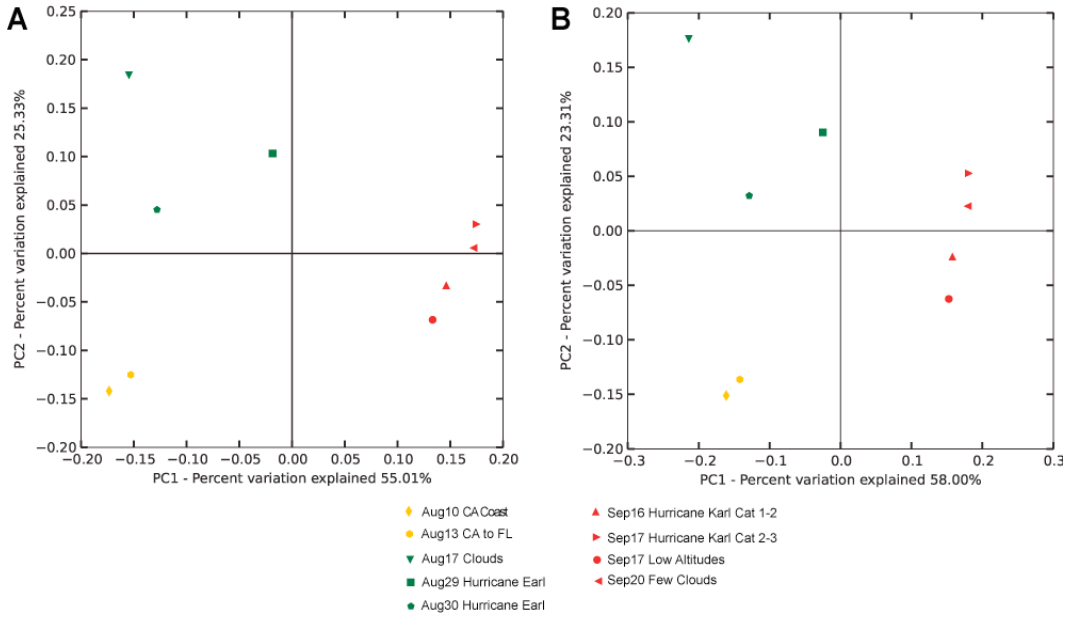


**Figure 2.6 Principal coordinates analysis (PCoA) of OTU abundance in all samples.** PCoA is based on the phylogeny-based beta diversity values estimated by the weighted UniFrac metric based on the OTU abundance data (Appendix A). The percent variation explained by each coordinate is shown on each axis. **Panel A** shows results using all sequences in each sample; **Panel B** is based on randomly subsampling 3,000 sequences per sample.

**2.4.3 Habitat of origin on Earth.** Airborne bacteria are thought to originate from different habitats on the Earth's surface (ocean, soil, freshwater, etc.). To gain quantitative insights into the habitat of origin of the microbes present in the GRIP campaign samples, we identified the best match of each sequence in the GreenGenes database (DeSantis, Hugenholtz et al. 2006) and analyzed the habitat of origin of the best matches when available. We also performed back trajectory analysis, which showed the route of the air masses for 5 d before sampling [e.g., continental vs. oceanic mass transport, (Fig. 2.7)], and contrasted this information to the habitat of origin of the SSU rRNA sequences.

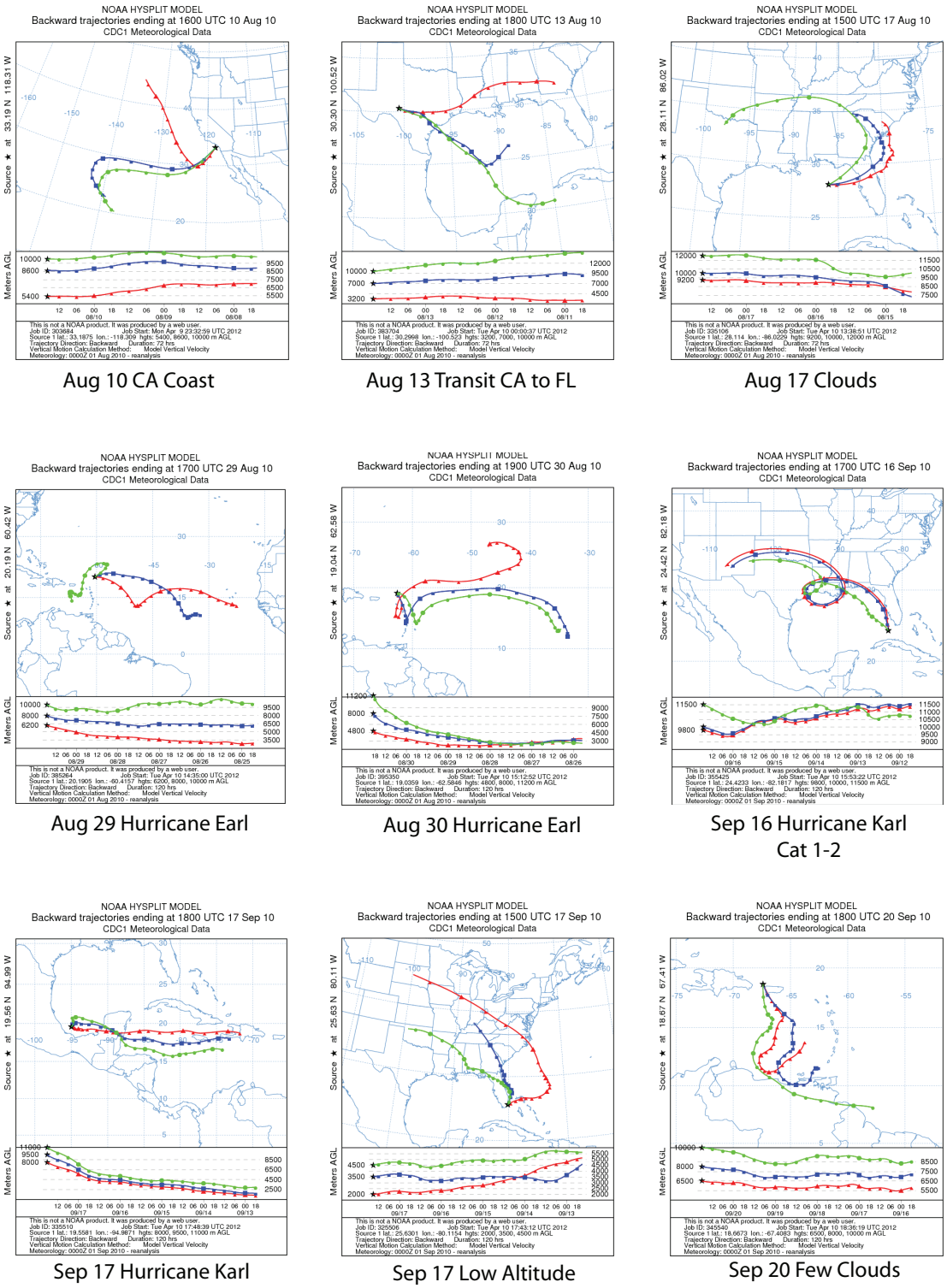


**Figure 2.7 Examples for back trajectory analyses for GRIP campaign flights.** Coordinates from the beginning, middle and end of the flight trajectory were selected to feed the back trajectory model for each flight. Using the NOAA HYSPLIT Model online service, back trajectories for each flight were analyzed to determine the route taken by the air masses during the last five days before sampling.

The results revealed that the organisms sampled originated from almost all habitats on Earth and that the hurricane samples had a higher abundance of marine bacteria (Fig. 2.8), which was consistent with back trajectory analysis and the geographic areas where the hurricanes developed. Furthermore, only in the hurricane samples did we

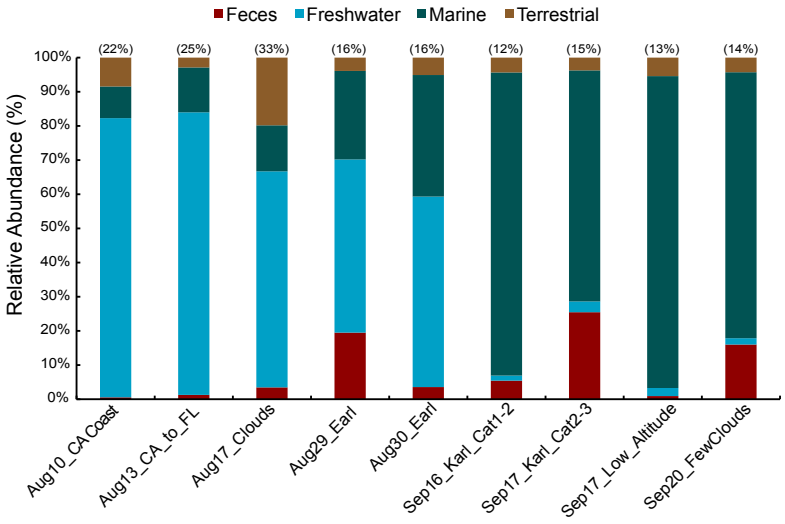


**Figure 2.8 Habitat of origin of the SSU rRNA gene sequences recovered in the GRIP samples.** Sequences were assigned to a habitat (see key) based on the source of isolation of their best match in the GreenGenes database. The graph represents the relative abundance of each habitat (vertical axis) for each sample (x axis). Numbers on the top denote the fraction of sequences that were assignable to a habitat for each sample. For a similar analysis that normalized for the differential representation of habitat among the reference GreenGenes sequences see Fig. 2.9.

observe a substantial signal of bacteria known to be associated with human and animal feces such as members of the *Escherichia* (38% in the first Hurricane Earl sample) or *Streptococcus* (26% in the second Hurricane Earl sample) genera, consistent with the passing of hurricanes over populated areas. However, at the resolution provided by our approaches, i.e., resolving phyla and genera well but species poorly, we could not confirm whether or not any of these bacteria represent pathogens. These results were reproducible when the differential representation of habitats in the GreenGenes Database was normalized (Fig. 2.9) or when the analysis was performed by using only one representative sequence for each OTU (as opposed to all sequences above) to avoid

potential amplification biases during sequencing that affected OTU relative abundance (*Discussion*). Our results were not always consistent with back trajectory analysis, and these cases were typically attributed to predictable processes or phenomena. For example, the California coast and Hurricane Earl samples were primarily characterized by oceanic transport, but we detected a relatively high abundance of freshwater and soil bacteria, respectively (Fig. 2.8). The former results are likely attributable to the long residence time of cells in the troposphere, longer than the 5 d period assessed by the back trajectory analysis, which is also consistent with the presence of several core OTUs in all GRIP samples. The presence of soil bacteria in Hurricane Earl samples is likely attributable to the strong winds from Africa, which preceded the period assessed by the back trajectory analysis and the development of the hurricane. These findings revealed that a "background" community was typically present in our samples and that back trajectory analysis can provide additional insight into the source region and long-range atmospheric transport of recently emitted bacteria, likely including disease-causing organisms.

## 2.5 Discussion

Our work shows that microbial cells, a majority of which appear to be bacterial, constitute an important component of the total super μm-sized particles in the mid/upper tropospheric air masses sampled (Fig. 2.2 *A* and Table 2.1).

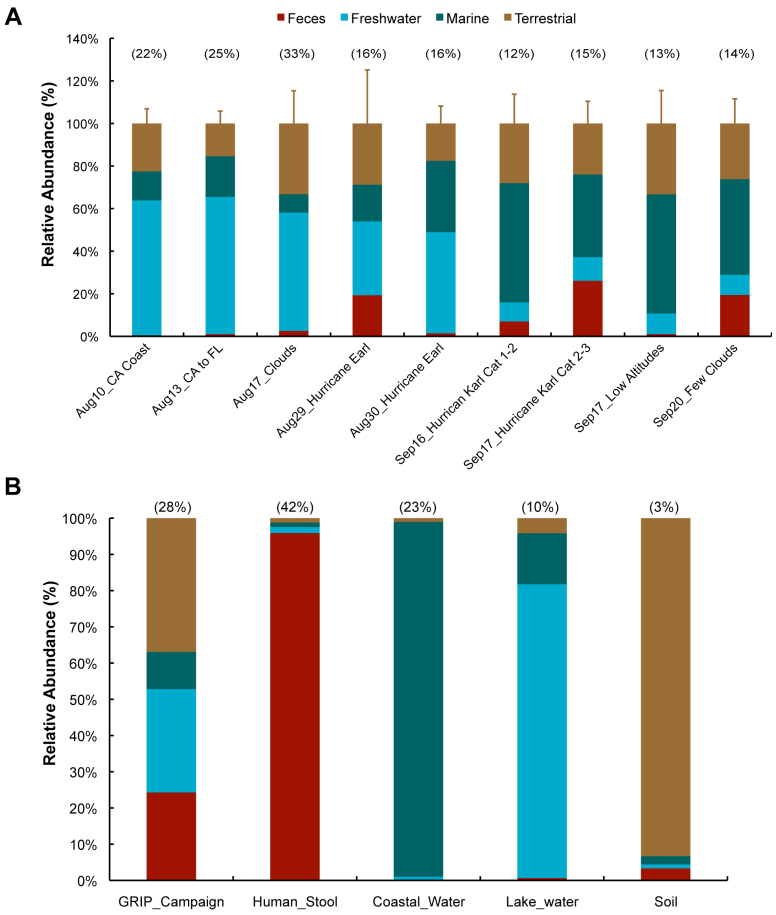


**Figure 2.9 Habitat assignment using the GreenGenes database. Panel A** is similar to Figure 3 except that the differential representation of habitats among the reference sequences from the GreenGenes database was normalized as described in the Materials and Methods section. In total, ten randomly drawn replicate datasets, each consisting of 1,000 GreenGenes sequences from each of the four habitats assessed, were obtained and used as the reference database to assign SSU rRNA gene sequences from our air samples to a habitat. Bars represent the average relative representation of the habitats (y-axis) in each of our samples (see labels on x-axis) based on the ten replicates. Error bars show the extent of variation observed (one standard deviation) among the ten replicates for the Terrestrial habitat as a representative example. **Panel B** shows the relative representation of each habitat for other publicly available datasets, i.e., Human_Stool (Momozawa, Deffontaine et al. 2011), Coastal_Water (Campbell, Yu et al. 2011), Lake_water and soil (Lauber, Hamady et al. 2009), compared to the average from our air samples based on a similar analysis. Note that the results for these samples were consistent with expectations, e.g., human stool sequences were mostly assigned to the "Feces" habitat, validating our approach. Numbers on the top denote the fraction of sequences of a sample that were assignable to a habitat (reference database).

Based on the variety of samples analyzed, the substantial presence of bacteria cells represents a robust feature of the troposphere and not a transient effect of large storm systems. Most of the airborne cells are viable (Fig. 2.2 *C*) and are large enough (0.25-1mm, Table 2.2) to support IN activity (DeMott, Prenni et al. 2010). Given also that many species (especially *Proteobacteria*) are known to be efficient nuclei for the

formation of water droplets and ice crystals, and that middle-to-upper troposphere (e.g., low temperature) clouds can be affected by low-activity IN (Murray, Wilson et al. 2010), airborne bacterial cells could influence cloud formation and precipitation more than previously thought. Furthermore, about 85% of the total SSU rRNA gene sequences that were recovered from the GRIP samples and were assignable to a habitat on Earth represented aquatic species (Fig. 2.8). This is consistent with previous studies showing that marine bacteria are more efficient CCN compared with bacteria from other ecosystems (e.g., soils) (Amato, Parazols et al. 2007, Junge and Swanson 2007). Assessing the CCN and IN potential of the cells in situ will provide for a more complete picture and allow parameterization of bacterial cell contribution to giant CCN and IN for use in cloud-resolving, regional, and global models. Our findings also reveal that tropical hurricanes aerosolize a large number of new cells and taxa, dramatically affecting the composition of the tropospheric communities for days after their passing (Fig. 2.5). Back trajectory analysis supported that air masses (and microbial cells transported with them) originated from lower altitudes and were brought aloft during the hurricanes. These findings indicate that long-range transcontinental transport of viable bacteria occurs, with potentially important implications for the biogeography of bacteria.

Our calculations also indicate that, at high altitudes, cells represent a much higher fraction of total particles than observed near the surface of Earth. This enrichment of cells at high altitudes cannot be solely attributed to the limitations of our methods or the difficulties associated with sampling air masses high in the troposphere (discussed below). Other factors such as faster growth of abiotic particles as CCN and/or IN

compared with cells and subsequent precipitation scavenging might be responsible for the patterns observed. It is also reasonable to hypothesize that different bacterial species show different CCN/IN; i.e., they are less affected by wet scavenging at lower altitudes. Consistent with this hypothesis, we found that *Gammaproteobacteria*, the most efficient IN bacteria known, did not make up a proportionally large part of the tropospheric communities (Table S3) compared to the near surface communities studied previously (Brodie, DeSantis et al. 2007, Bowers, Lauber et al. 2009, Bowers, Sullivan et al. 2011). Furthermore, model simulations have shown that CCN-active microorganisms have shorter residence time in the atmosphere than those that are CCN-inactive (Burrows, Butler et al. 2009). This hypothesis is still consistent with the idea that airborne microbial cells significantly influence cloud formation and precipitation at medium-to-high altitudes because the prevailing in situ conditions favor the nucleation of ice by even relatively inefficient IN particles. However, more observations and modeling studies are clearly required to support the above. Our findings do highlight that the life cycle of bioaerosols may differ from that of abiotic particles and underscore the need to better understand emissions, transport, and removal of bioaerosols.

The presence of 17 OTUs in all samples of our study (core microbiome), even those taken after the impact of the two hurricanes, implies that these OTUs represent organisms that have developed mechanisms - such as mechanisms to cope with UV, desiccation and large concentrations of oxidants (OH, $O_3$, $H_2O_2$) - that have allowed them to survive long periods in the atmosphere. Previous literature indicates that this hypothesis may be true. For example, *Afipia* sp. was the most abundant core group in our

samples, especially before the pass of the hurricanes. This group is commonly found in aquatic environments and is known to use dimethyl sulfone ($DMSO_2$) as a sole carbon source (La Scola, Barrassi et al. 2000, Moosvi, Pacheco et al. 2005). $DMSO_2$ represents an intermediate of the oxidation of dimethyl sulfide (DMS), which is commonly found in the marine atmosphere (Davis, Chen et al. 1998). Thus, *Afipia* sp. could potentially survive in clouds by using the available carbon compounds present in the atmosphere. Previous studies have also found members of the *Bradyrhizobiaceae* family (to which *Afipia* sp. is assigned) to compose around 10% of the total community in samples collected from cloud water (Bowers, Lauber et al. 2009), which is consistent with our findings that this group is ubiquitously present in the atmosphere. Similar to *Afipia* sp., *Oxalobacteraceae* and *Methylobacteriu*m, two other core groups, can also use C1-C4 compounds that are ubiquitously present in the atmosphere, and concentrated (in the millimeter range) in cloud water.

The results presented here are largely consistent with existing knowledge but with a few notable exceptions. For example, Harrison et al. estimated the average bacterial cell concentration in near-surface air over coastal ecosystems to be $7.6x10^4$ cells $m^{-3}$ (Harrison, Jones et al. 2005). Bacterial cell concentration in the cloud-free, high-altitude sample collected during the GRIP campaign based on our microscopy-based counts is about twofold lower (it is also likely that the real difference is even greater because the methods used in the previous study are more comparable to our qPCR than the microscopy methods). However, the previous study was conducted at sea level whereas GRIP samples were collected at 8 km or higher in the troposphere, and aerodynamic

particle sizer analysis showed that coarse-mode particle concentration at these altitudes is at least an order of magnitude lower compared with at the Earth's surface (Fig. 2.3). Principal Coordinates Analysis (PCoA) also revealed that the microbial communities in the GRIP samples were much more similar to those associated with air and snow collected at the top of high mountains compared with oceanic or soil communities (Fig. 2.5 B). These results show that sampling air at the top of mountains or snow on the ground may reflect the medium-to-upper troposphere. Despite the similarities between ground and snow samples and the ones collected in this study, however, there are several striking and profound differences that suggest a very dynamic and diverse tropospheric microbiome that merits considerable more attention. For example, substantially fewer OTUs were found in this study compared with samples collected at 3.2 km at the top of Colorado mountains (Bowers, Lauber et al. 2009), indicating that not all microbial cells can apparently reach or survive high-altitude conditions. Furthermore, contrary to previous surveys of near-surface atmospheric samples (Brodie, DeSantis et al. 2007, Bowers, Lauber et al. 2009, Bowers, Sullivan et al. 2011), *Gammaproteobacteria* did not make up a proportionally large part of the tropospheric communities sampled. These findings might reflect the influence of atmospheric processes and transport of the bacterial cells because several members of *Gammaproteobacteria,* for example, are known to be CCN- and IN-active.

The high abundance (>40% of the total community) of a few OTUs - i.e., the *Afipia* sp. in the California coast and the transit flight samples and the *Burkholderiales* order in Hurricane Karl samples - was somewhat surprising because the conditions

prevailing in the atmosphere are not expected to favor the high abundance or fast growth of a few individual species. Furthermore, back trajectory analysis was not always consistent with the high abundance of these OTUs. For example, the majority of sequences that made up the *Afipia* sp. OTU in the California coast sample best matches to sequences of freshwater organisms in GreenGenes database; yet, the air masses sampled in this case were influenced primarily by oceanic, not continental transport. Therefore, the high abundance of a few OTUs might be due, in part, to experimental artifacts and noise. For example, we (Oh, Caro-Quintero et al. 2011) and others (Zhou, Wu et al. 2011) have noted that amplification biases can affect the results of amplicon pyrosequencing. Due to these limitations, we also performed a large part of our analyses using OTU presence/absence as opposed to relative abundance (Fig. 2.8), which did not differentiate our conclusions substantially. Furthermore, the *Afipia* sp. and *Burkholderiales* OTUs were present in all samples (core OTUs) and previous studies have also frequently recovered these groups from air samples (Bowers, Lauber et al. 2009, Bowers, McLetchie et al. 2011). Thus, the qualitative trends in OTU distribution and relative abundance in different samples reported here should be robust.

It is technically challenging to achieve high sampling flow rates and filter large quantities of air at high altitudes. Our experimental design represented a compromise between obtaining high volumes of air and having a controlled, enclosed system to avoid contamination. The low biomass collected, and as a result low DNA yields, imposed additional challenges for cell counting and molecular work (qPCR and SSU rRNA gene amplicon sequencing). Accordingly, at least some variation in the results obtained is

presumably attributable to experimental noise. However, this noise was not high enough to confound our results as evidenced by several metrics, such as the at least one order of magnitude higher cell counts in field samples vs. blanks; the consistency between qPCR, SSU rRNA gene sequencing, and microscopy results; the high similarity in community composition among related samples [e.g., same hurricane (Fig. 2.5 *A*)] but not among unrelated ones (different hurricanes); and several results that were consistent with expectations and/or previous literature (e.g., higher cell counts in low altitude and cloud samples compared with high altitudes and no clouds, respectively).

The results presented here represent a culture-independent analysis of the microbial communities of the middle-to-upper troposphere above the oceans and advance our understanding of the composition of these communities and their shifts over time, space, and environmental perturbations caused by tropical storms or hurricanes. This information is important for modeling the dispersal of microbial diseases and for determining which microbes show limits (or no limits) in terms of dispersion through the atmosphere. Our results also indicate that airborne microbial cells may quantitatively be more important for cloud formation and precipitation than previously anticipated. Clearly, more attention should be given to these microbes and their role in the atmosphere compared with what has been accomplished to date. Microbes are known to actively contribute to, if not drive, the geochemistry in all habitats on Earth. The results reported here indicate that airborne microbes may have a similarly important role in the (bio)chemistry of the atmosphere and the hydrological cycle.

| Date | Flight description | Sampling region | Air filtered ($m^3$) | Average altitude (km) | Bacterial cells / $m^3$ (% viable) | 0.25-1.0µm diameter aerosol /$m^{-3}$ | Bacterial contribution (%) |
|---|---|---|---|---|---|---|---|
| 08/10/2010 | Off coast; pristine conditions | Eastern Pacific, California | 2.88 | 9 | ND | ND | ND |
| 08/13/2010 | Transit flight from California to Florida | Intercontinental (USA) | 5.04 | ND | ND | ND | ND |
| 08/17/2010 | Clouds | Gulf of Mexico | 2.22 | 10 | $1.23x10^5$ *(96)* | $0.57x10^6$ | 21.6 % |
| 08/29/2010 | Hurricane Earl | St. Croix-Fort Lauderdale | 5.00 | 10 | ND | $0.2x10^6$ | ND |
| 08/30/2010 | Hurricane Earl | St. Croix-Fort Lauderdale | 7.40 | 10 | $0.76x10^5$ *(100)* | $0.16x10^6$ | 47.5 % |
| 09/16/2010 | Hurricane Karl Category 1-2 | Bay of Campeche | 14.86 | 10 | $3.04x10^5$ *(97)* | $0.11x10^6$ | 276 % |
| 09/17/2010 | Hurricane Karl Category 2-3 | Mexico | 13.67 | 10 | $1.24x10^5$ *(60)* | $0.13x10^6$ | 95.4 % |
| 09/17/2010 | Low altitude | Fort Lauderdale | 2.83 | 3 | $2.11x10^5$ *(96)* | $5.89x10^6$ | 3.6 % |
| 09/20/2010 | Few Clouds | Fort Lauderdale – St. Croix | 2.18 | 9 | $0.36x10^5$ *(81)* | $0.36x10^6$ | 10.0 % |

**Table 2.1 The GRIP research flights conducted for this study.** Altitude measurements (5th column) represent the average during the bioaerosol collection time. The percent of live cells, the great majority of which was bacteria (6th column, parentheses), was estimated based on live/dead stained cells from sixteen 90x90 $\mu m^2$ fields per sample. Total aerosols in the size range 0.25 to 1µm, including cells as well as abiotic particles (7th column), were determined using an UHSAS instrument. Bacterial contribution to total aerosols (8th column) was estimated assuming that all cells observed in the sample (6th column) were bacterial and 0.25 to 1µm in diameter. Note that one of the Hurricane Earl samples showed >100% bacterial contribution, which is presumably attribute to the fact that not all cells in the sample were actually 0.25 to 1µm in diameter (Table S2) and the experimental noise associated with the analysis of low biomass samples and the methods employed. ND – not determined.

| | Fraction of the total cells in the sample (%) | | | | | |
|---|---|---|---|---|---|---|
| **Size (µm)** | **Aug_17 Clouds** | **Aug_30 Hurricane Earl** | **Sep_16 Hurricane Karl** | **Sep_17 Hurricane Karl** | **Sep_17 Low Altitude** | **Sep_20 Few Clouds** |
| **0.2** | 6.7 | 0.0 | 5.1 | 6.7 | 0.0 | 9.5 |
| **0.3** | 21.7 | 4.5 | 11.2 | 26.7 | 25.0 | 0.0 |
| **0.4** | 16.7 | 9.1 | 12.4 | 46.7 | 16.7 | 28.6 |
| **0.5** | 15.0 | 13.6 | 11.8 | 0.0 | 8.3 | 9.5 |
| **0.6** | 3.3 | 9.1 | 19.1 | 0.0 | 0.0 | 0.0 |
| **0.7** | 13.3 | 18.2 | 14.0 | 6.7 | 8.3 | 14.3 |
| **0.8** | 10.0 | 18.2 | 3.9 | 0.0 | 16.7 | 0.0 |
| **0.9** | 3.3 | 0.0 | 6.2 | 0.0 | 8.3 | 0.0 |
| **>1.0** | 10.0 | 27.3 | 16.3 | 13.3 | 16.7 | 38.1 |

**Table 2.2 Size distribution of the cells in each sample as determined by microscopy**. Samples were analyzed as described in the Material and Methods section and cell size was determined using the AxioVision Rel. 4.8 software.

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# CHAPTER 3

## Measuring the cloud condensation nuclei activity of bacterial cells: implications for atmospheric processes

### 3.1 Abstract

The abundance of bacterial cells in the atmosphere can reach relatively high numbers, but the role of airborne cells in atmospheric chemistry, cloud formation, and precipitation remains to be quantified. For instance, airborne cells can serve as efficient cloud condensation nuclei (CCN), participating in water formation and precipitation. A few studies exist that have measured the CCN activity of different bacterial cells, but there is poor understanding of the underlying cellular components that contribute to this activity and how the activity is related to bacterial cell hygroscopicity and the physiological state of the cell. The objective of this study was to determine whether the affinity of different bacterial cells to water alone is sufficient to explain cell ability to act as CCN. To this end, we collected samples from rainwater and ambient air at different locations (urban cities and rain forest) and altitudes (~10 km and surface air). Over 20 bacterial isolates were obtained from these samples using different minimal and rich media, and were identified based on sequencing of the 16S rRNA gene. The hygroscopicity of different bacterial isolates was evaluated based on their contact angle with water. A wide range of contact angles was observed among our isolates, ranging from very hydrophilic to very hydrophobic; the majority of the isolates however were found to be hydrophilic. The CCN activity of each isolate was studied by introducing aerosolized bacteria into a

continuous flow stream-wise thermal gradient CCN counter. Hydrophilic bacteria were found to have a critical supersaturation of 0.1% compared to hydrophobic bacteria with a critical supersaturation of 0.2% or higher. These supersaturation conditions are often encountered in the atmosphere of several parts of the planet. Therefore, our results indicate that hydrophilic bacteria could influence cloud formation and precipitation in these geographic areas.

## 3.2 Introduction

Aerosols play an important role in the formation of clouds and precipitation. Organisms such as bacteria, fungi, spores, and viruses, when injected into the atmosphere are known as primary biological aerosols. Based on their sizes, aerosols in general, are divided into three different modes: aitken mode (0.01 - 0.1 μm), accumulation mode (0.1 - 1 μm), and coarse mode ( > 1μm) (Seinfeld and Pandis 2012). Bacterial cells tend to be part of the coarse mode, and at high altitudes could represent up to 20% of the total number of the particles in this mode, at least in air masses affected by hurricane systems (DeLeon-Rodriguez, Lathem et al. 2013). Previous studies isolated bacteria from cloud water (Bauer, Giebl et al. 2003, Amato, Parazols et al. 2007, Amato, Parazols et al. 2007), and Amato (2010) suggested that cloud droplets could represent an oasis for bacteria that are present in the atmosphere, providing nutrients and protection against UV radiation (Amato 2010). Bacterial cells can also promote such favorable conditions for their survival in the atmosphere and participate in cloud formation processes by serving

as cloud condensation nuclei (Franc and DeMott 1998, Bauer, Giebl et al. 2003). Cloud formation occurs when water vapor condenses over a particle (e.g., aerosols) and the relative humidity is over 100% (referred to as water vapor supersaturation). Aerosols in coarse mode are known to influence the giant CCN (gCCN; > 5 μm) population. Larger concentrations of gCCN are thought to increase precipitation in warm continental clouds (Kuba and Takeda 1983, Möhler, DeMott et al. 2007).

Different bacterial species show varied CCN activities but what cellular constituents and/or physiological adaptations underlie this variation remain poorly understood. Only a limited number of studies have measured the CCN activity of bacterial cells isolated from plants, air, or cloud water (Franc and DeMott 1998, Bauer, Giebl et al. 2003). Previous studies agreed in that bacterial cells are good CCN and better than Kelvin particles (a perfectly wettable non-soluble particle) of the same size. For instance, Bauer et al. measured the CCN activity of bacteria isolated from aerosol and cloud water samples collected in the Alps using a CCN static thermal diffusion chamber (Bauer, Giebl et al. 2003). From all the isolates obtained during this study only three species where tested for CCN activity, which were identified as *Arthrobacter agilis*, *Sphingomonas echinoides*, and an unknown taxon. Close to 100% of the measured cells in this study were found to activate at supersaturations between 0.07 to 0.11%. These results indicate that bacterial cells are better CCN than a Kelvin particle of similar size, which is expected to activate at 0.3% critical supersaturation. Other studies have reported much lower CCN activities for different bacterial species, indicating that CCN activity may be species-dependent. For instance, Franc and DeMott measured the CCN activity of *Erwinia carotovora* isolates using a thermal gradient diffusion chamber (TGDC) (Franc

and DeMott 1998). This study showed that 25 – 30% of the total *E. carotovora* cells activate at 1% supersaturation (101% relative humidity). Both studies suggested that bacterial cells are efficient CCN, enough to influence the gCCN population, therefore, they have potential to affect precipitation of warm clouds (assuming enough cells are present in-situ). Nonetheless, the differences in the results of these previous studies are remarkable, and it could be attributed to the different methods used.

Bauer et al. hypothesized that the membrane composition of bacterial cells is responsible for their CCN activity (Bauer, Giebl et al. 2003). The bacterial cell membrane is a physical characteristic that is well-understood, at least for model organisms, and is important in cell-cell or cell-surface adhesion. Several studies have measured the degree of cell hygroscopicity of different microorganisms (Weiss, Rosenberg et al. 1982, Busscher, Weerkamp et al. 1984, Minagi, Miyake et al. 1986, Van der Mei, Weerkamp et al. 1987, Van der Mei, Bos et al. 1998, Van der Mei, Van de Belt-Gritter et al. 2003). Hygroscopicity is typically reported as the contact angle between a liquid and the bacterial lawn. However, no study to date has assessed the degree of hygroscopicity of bacterial cells collected from atmospheric samples and its relation to the CCN activity of the cells. Further, previous studies have shown that cell membrane lipid composition, and hence, potentially the degree of cell hygroscopicity, changes with growth temperatures. In particular, homeostatic changes occur when cells are exposed to lower temperatures, favoring more unsaturated acyl side chains in the cell membrane, thereby increasing membrane fluidity to counter the cold temperatures. Hence, it is likely that, under atmosphere-like (e.g., cold temperature) conditions, the CCN activity of bacterial cells differs compared to room conditions.

This chapter aimed to understand and quantify how membrane hygroscopicity affects CCN activity of bacteria isolated from atmospheric samples and grown under different conditions, by measuring their contact angle and CCN activity. Our working hypothesis was that bacterial cells that are more hygroscopic tend to be better CCN compared to less hygroscopic cells and that CCN activity differs at different physiological states of the cell, e.g., exponential vs. stationary phase. To test this, a stream-wise thermal gradient cloud condensation nuclei chamber (Roberts and Nenes 2005) was used for measuring CCN activity of cells characterized by different contact angles, coupled to several pre-processing steps to exclude contamination and CCN activity of abiotic particles present in the water or growth media. Specifically, we tested whether or not bacteria cells with contact angle smaller than 40° (i.e., hydrophilic) have a lower critical supersaturation compared to those with contact angles greater than 50° (i.e., hydrophobic).

## 3.3 Materials and Methods

### 3.3.1 Bacterial isolates from atmospheric samples

Atmospheric samples were collected from rainwater and clean air from several locations across the continental USA and Puerto Rico (Table 3.1). The rainwater was collected using a previously sterilized funnel and stored in a sterile 50 mL falcon tube. Clean air samples were collected during the National Aeronautics and Space Administration (NASA) Genesis and Rapid Intensification Processes (GRIP) campaign in

summer 2010. The bioaerosols were collected on cellulose nitrate membranes (0.2 μm pore size and 47 mm diameter; Whatman, GE Healthcare; Pittsburgh, PA). All samples were stored at 4°C until processing, as described previously (DeLeon-Rodriguez, Lathem et al. 2013). Bacterial cells were detached from the membrane by placing it in 10 mL of 1X Phosphate-Buffered Solution in a shaker at 220 rpm at 10° C for 20 minutes. All the samples were used to inoculate four different media: Luria Bertani agar (10 g/L of tryptone; 5 g/L of sodium chloride; 5 g/L yeast extract), Difco™ R2A, ¼ Difco™ TSA, and Stanier's mineral salt broth (Gibson, Cardini et al. 1970) with lactate or glucose (20 mM) and incubated at room temperature until colonies were visible. Picked colonies were transferred four times to new media plates to assure single isolates were obtained. Cycloheximide (50 μg/mL) was added to the media to avoid fungal growth except to the ¼ Difco™ TSA growth media. Glycerol stocks were made for long term storage by mixing an overnight culture with glycerol (final concentration 20%) and storing it at -80°C. Sequencing of the 16S rRNA gene was employed to identify the isolates using universal primers (8F-Forward 5' – AGAGTTTGATCATGGCTCAG – 3' and 1492R-Reverse 5' – GGTTACCTTGTTACGACTT – 3'). PCR reactions were performed using 1.25U of Ex Taq™ enzyme (Takara), 1X reaction buffer, 1.0 μM of each primer and 1.0 mM of dNTPs. PCR conditions were set as: initial denaturation at 98°C for 2:10 min, followed by 30 cycles at 98°C at 10 s, 55°C for 45 s, and 72°C for 2:10 min, with a last elongation at 72°C for 2:10 min.

### 3.3.2 Growth and contact angle measurements of isolates

The growth rate of each isolate was measured using optical density (OD) over a 12 h period and a spectrophotometer (HACH DR 2800; Loveland, CO). For this, isolates were grown in LB broth starting from an overnight inoculum and OD was measured every hour. Bacterial cells were collected during late exponential phase (OD = 0.4) for quantification using flow cytometry. Bacterial cells were fixed with formalin after collection, samples were diluted 1:10 and stained with SYTO-13 (5 μg/mL), during 10 min.

For the purpose of the contact angle experiment, cells were harvested during two times in the growth curve, at late exponential phase (OD = 0.4), and at late stationary phase, in duplicates. Cells were washed twice with deionized water (di-water) for 20 min at 4500 rpm. Subsequently, cells were filtered through a 0.45 μm membrane to a cell density of $10^8$ cells per $mm^2$ (Busscher, Weerkamp et al. 1984). The resulting bacterial lawn was dried with two different methods: (1) air-dried at room temperature in the laminar flow (~ 27% relative humidity), and (2) the filter was placed in a silica gel drier at room temperature (~ 4% relative) humidity for 30 min. Once the filter dried, it was cut in strips and attached to a glass slide using double-sided tape. The contact angle formed by water on top of the bacterial lawn was measured using the KSV CAM-200 goniometer. This instrument consists of a light source, a high-resolution camera, and a sample stage-holder. Using a micropippetor, 2 μL of di-water was placed on top of the bacterial lawn and 10 frames were taken every 2 s. Contact angle was calculated for each frame using the KSV CAM-200 software and reported the average angle for both sides of each droplet. The data was plotted using a linear regression and the y-intercept value was

reported as the contact angle for each bacteria isolate (Busscher, Weerkamp et al. 1984). This was done to avoid errors in lag time recording the measurements. For each of the isolates, more than three droplets were measured for each duplicate sample.

### 3.3.3. CCN activity measurements

Bacterial isolates were grown overnight and washed twice with milli-Q water (two times the volume) and re-suspended in cell culture water. Seven isolates were selected to measure their CCN activity, in triplicates. CCN activity was measured in a system that consisted of three parts: aerosol generation, small particle removal, and particle counter and CCN activity measurement (Fig 3.1). Aerosols were generated from a liquid solution using a simple large volume nebulizer (Teleflex, Inc.). Liquid droplets were dried using two silica gel diffusional driers, maintaining a relative humidity below 5% or four silica gel diffusional driers, with a relative humidity of ~0% at the end of the line. A Differential Mobility Analyser (DMA;TSI Inc., Model 3081 Long DMA) with a Kr-85 charger, was used to select aerosols between 800nm to 1000nm in diameter. The sheath flow to sample flow ratio was 2.0 to 0.5 lpm. Particle concentration was recorded during the experiment using a condensation particle counter (CPC; TSI 3010). CCN activity was measured using a stream-wise thermal gradient cloud condensation nuclei chamber (Roberts and Nenes 2005). The sample flow was kept constant at 0.5 and 0.3 lpm for comparisons. The sample traveled through a wetted column, which is set at different supersaturation (0, 0.1, 0.15, 0.2, 0.4, 0.5, 0.6%) for 20 min each. An Optical Particle Counter (OPC) detects CCN active particles (water droplets) at the end of the column and the droplet size distribution is recorded.

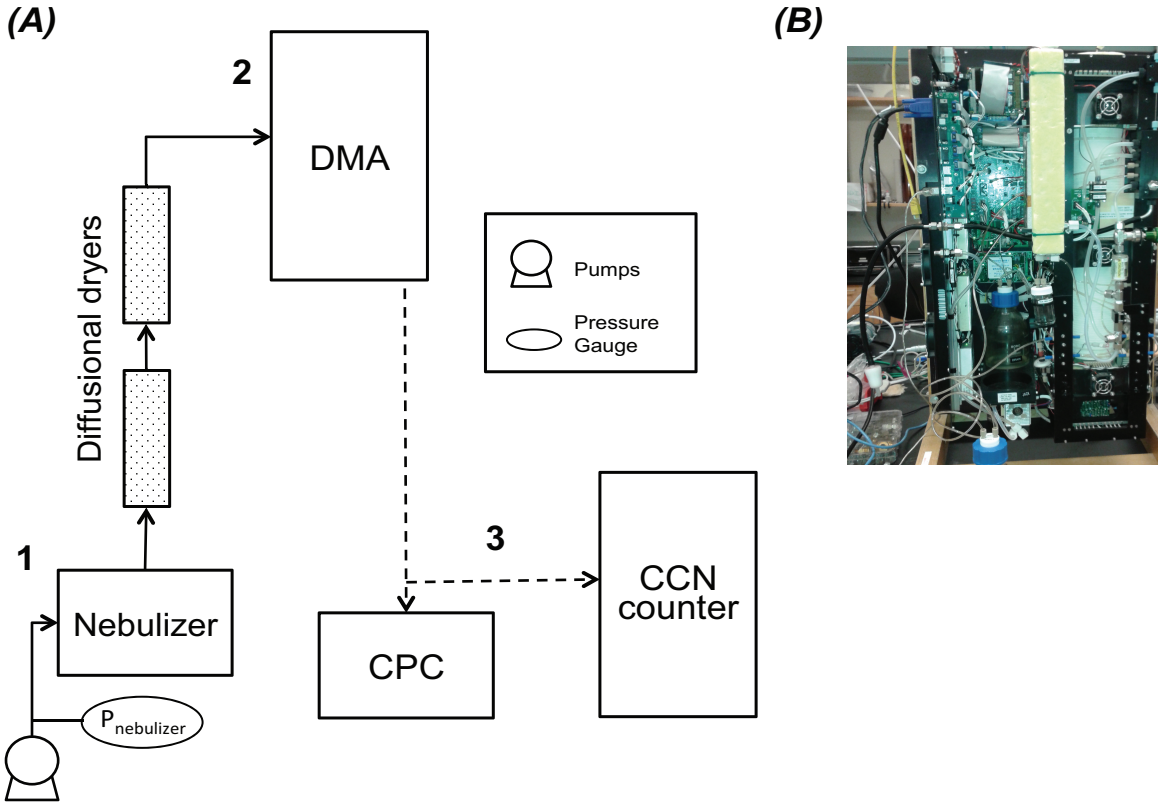


**Figure 3.1 Schematic diagram of CCN instrumentation setup.** The setup is divided in three sections: (1) aerosol generation; (2) size selection; and (3) particle and CCN quantification (A). A picture of the CCN counter instrument is shown in panel B.

### 3.3.4 Fluorescent microscopy

Fluorescent microscopy was used to determine the size of the bacterial cells used for the CCN experiments as well as to detect and confirm the presence of intact cells in the sample flow. Bacterial cell sizes were measured using a sample from the bacterial suspension used to generate the aerosols and a filter that was collected before the inlet of the CCN chamber (the latter was used to confirm the efficiency of bacterial cells to travel through the setup) on an Axio Vision Observer D1 (Zeiss). All samples were stained using 4',6-diamidino-2-phenylindole (DAPI; 5 µg/ml) and incubated at room temperature in the dark for 10 min.

### 3.3.5 Global plots of in-cloud $S_{max}$

The regional distribution of in-cloud maximum supersaturation was estimated using the Community Atmosphere Model (CAM5.1), which is a state-of-the-art atmospheric general circulation model with fully coupled aerosol-cloud interactions (Liu, Easter et al. 2012). In the simulations presented here, we used the model configured with a finite volume dynamic core, a horizontal resolution of 1.9° × 2.5°, and 30 pressure levels in the vertical. The 3-mode version of the modal aerosol module (MAM3) was used, which considers aerosol sulfate, ammonium, nitrate, primary organic matter, secondary organic aerosol, black carbon, sea salt, and dust; particles are distributed into Aitken, accumulation, and coarse lognormal modes with prescribed geometric standard deviation. The MAM3 is coupled to a double moment cloud microphysics scheme. Particles can be removed by wet removal mechanisms, or regenerated to interstitial aerosol after cloud droplets evaporate. Activation of aerosol to cloud droplets is calculated with a mechanistic aerosol activation parameterization (Fountoukis and Nenes 2005). This parameterization computes the maximum supersaturation attained in an air parcel by explicitly considering the competing effects of water vapor supersaturation depletion by condensation on the growing droplet population, and the generation of water vapor supersaturation by adiabatic expansion and cooling. These competing factors finally determine the maximum supersaturation that is attained in this ascending parcel, under the conditions of temperature, pressure, humidity, and aerosol characteristics. Simulations were performed for current-day and preindustrial emissions of aerosol

precursors (Lamarque, Bond et al. 2010), with climatological sea surface temperatures and ice cover.

## 3.4 Results

### 3.4.1 Sample collection, bacteria isolation and identification

Bacteria from air and rainwater were isolated from different locations in the continental U.S. and Puerto Rico. Air samples were collected as part of the NASA GRIP campaign. Two of the samples were collected in the area of the Caribbean (GRIP) and one off the coast of California (GRIP). Rainwater samples were collected in two urban zones (Atlanta, GA and Carolina, PR) and one forested zone (El Yunque National Rain Forest, Luquillo, PR) (Table 3.1). We obtained approximately 10 to 15 mL of rainwater from each sample. Samples from forested zones were collected in area with no vegetation above the collection site.

In total, 29 bacterial isolates were obtained from all samples (Table 3.2). Isolates, which were identified as members of the *Pseudomonas* sp., *Bacillus* sp., *Staphylococcus* sp., *Curtobacterium* sp., *Erwinia* sp., and *Microbacterium* sp. *Pseudomonas* sp. and *Erwinia* sp. based on 16S rRNA gene sequencing, were more predominant in the rain forest samples. In contrast, isolates collected at high altitudes during the NASA GRIP campaign were identified as *Bacillus* sp. and *Curtobacterium* sp., while *Staphylococcus* sp. and *Microbacterium* sp. isolates were found only in samples collected in urban areas (Carolina, Puerto Rico).

**Table 3.1** Sample information. 'XXX' refers to the 3-digit identification number used for the isolates obtained from each sample.

| Sample ID | Location | Collection Date | Type of sample | Environment |
|---|---|---|---|---|
| ATL13_XXXR | Atlanta, GA | 08/07/13 | Rain | Urban |
| CARPR13_XXXR | Carolina, PR | 08/10/13 | Rain | Urban |
| RFPR13_XXXR | Luquillo, PR | 08/11/13 | Rain | Forested |
| TEST_XXX | California | 08/10/10 | Air | Urban/Ocean |
| F7_XXX | Caribbean | 08/24/10 | Air | Ocean |
| F8_XXX | Caribbean | 08/28/10 | Air | Ocean |

### 3.4.2 Hygroscopicity of bacterial isolates based on contact angle measurements

Contact angles were measured after placing a sessile water drop on top of the bacterial lawn (as describe in Section 3.3.2). Results showed that most bacteria were hydrophilic with a contact angle around 30° and only 2 isolates had a contact angle over 80° (CARPR_006R and ATL13_016R; Fig 3.2A). All *Pseudomonas* isolates had contact angles within the 19-33° range. In contrast, both *Staphylococcus* isolates had very different contact angles, 31° (ATL13_012R) and 98° (CARPR13_006R). In the case of the *Bacillus* isolates, 2

**Table 3.2 Isolates identification and information.** ID refers to the isolate identification name. Best Match is based on homology search of 16S rDNA gene sequence against the nr database of NCBI at the genus level. KB refers to the King's B agar test to determine if the isolate is *P. syringae* or not. ND = Not determined.

| ID | Location | Source | Best Match | Gram Stain | KB agar test |
|---|---|---|---|---|---|
| ATL13_001R | Atlanta, GA | Rainwater | *Pseudonomas* | - | - |
| ATL13_002R | Atlanta, GA | Rainwater | *Bacillus* | + | - |
| ATL13_012R | Atlanta, GA | Rainwater | *Staphylococcus* | - | - |
| ATL13_016R | Atlanta, GA | Rainwater | *Microbacterium* | + | - |
| CARPR13_003R | Carolina, PR | Rainwater | *Pseudonomas* | + | - |
| CARPR13_004R | Carolina, PR | Rainwater | *Microbacterium* | + | - |
| CARPR13_006R | Carolina, PR | Rainwater | *Staphylococcus* | - | - |
| CARPR13_011R | Carolina, PR | Rainwater | ND | | - |

| | | | | | |
|---|---|---|---|---|---|
| F7_001 | Caribbean | Air | *Bacillus* | + | - |
| F8_003 | Caribbean | Air | ND | | - |
| RFPR13_001R | Luquillo, PR | Rainwater | *Pseudonomas* | - | - |
| RFPR13_002R | Luquillo, PR | Rainwater | *Pseudonomas* | - | - |
| RFPR13_003R | Luquillo, PR | Rainwater | *Pseudonomas* | - | + |
| RFPR13_005R | Luquillo, PR | Rainwater | *Pseudonomas* | - | - |
| RFPR13_006R | Luquillo, PR | Rainwater | *Erwinia* | - | - |
| RFPR13_007R | Luquillo, PR | Rainwater | *Pseudonomas* | - | + |
| RFPR13_008R | Luquillo, PR | Rainwater | *Erwinia* | - | - |
| RFPR13_009R | Luquillo, PR | Rainwater | *Pseudonomas* | - | + |
| RFPR13_010R | Luquillo, PR | Rainwater | *Pseudonomas* | - | - |
| RFPR13_012R | Luquillo, PR | Rainwater | *Pseudonomas* | - | + |
| RFPR13_013R | Luquillo, PR | Rainwater | *Pseudonomas* | - | + |
| RFPR13_014R | Luquillo, PR | Rainwater | *Pseudonomas* | - | - |
| RFPR13_015R | Luquillo, PR | Rainwater | *Pseudonomas* | - | + |
| RFPR13_016R | Luquillo, PR | Rainwater | *Pseudonomas* | - | + |
| Test_002 | California, USA | Air | *Bacillus* | + | - |
| Test_004 | California, USA | Air | *Bacillus* | + | - |
| Test_005 | California, USA | Air | *Curtobacterium* | + | - |
| Test_007 | California, USA | Air | *Curtobacterium* | + | - |
| Test_008 | California, USA | Air | *Curtobacterium* | + | - |

**Table 3.2 (Continued) Isolates identification and information.** ID refers to the isolate identification name. Best Match is based on homology search of 16S rDNA gene sequence against the nr database of NCBI at the genus level. KB refers to the King's B agar test to determine if the isolate is *P. syringae* or not. ND = Not determined.

isolates had a contact angle of 52° (TEST_002 and TEST_004), while one of them had a lower angle, 27° (F7_001). The isolate with the lowest contact angle, 5°, was identified as *Pseudomonas* sp. isolated from rainwater collected in an urban zone (CARPR3_003R).

Four isolates were selected to test if growth phase affect their hydrophobicity. For this, cells were collected during late logarithmic and late stationary phase and the contact angle was measured as described above and found to be similar (Fig 3.2B). In addition, a second drying method was tested using silica beads in order to get a lawn as dried as possible for the CCN measurements. Differences in contact angles were observed for all four isolates during the drier conditions, two of them changed from hydrophilic to

hydrophobic and the other two stayed hydrophilic but showed high larger angles (less hydrophilic). One of the isolates increased from 27° to 50° after drying the lawn, once the lawn was wetted again the angle decreased to 19° (Fig 3.2B). The other isolate changed from 54° to 98°, going back to 67° after rewetting the lawn.

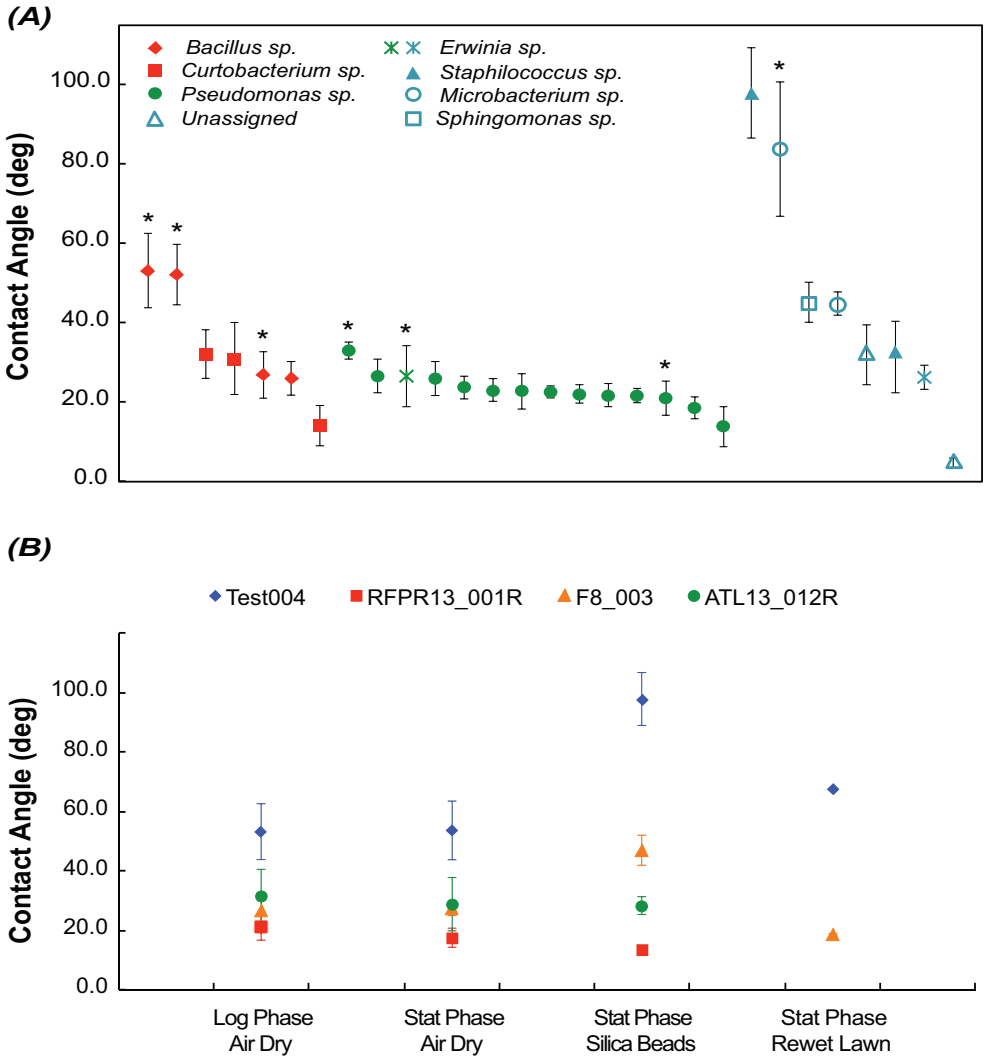


**Figure 3.2 Measuring cell membrane hydrophobicity of bacterial isolates.** (**A**) Contact angle measurements of bacterial isolates recovered from atmospheric samples. Each point represents one isolate. Taxonomy identification was determined based on best match analysis of 16S rRNA gene sequences (see figure key). Error bars represent standard deviation of at least three replicates (water droplets). Asterisks denote the isolates used in CCN experiments. Colors denote: red, air samples from California collected during the GRIP campaign; green, rainwater from El Yunque National Rain Forest in Puerto Rico (PR); blue, rainwater from urban areas in Atlanta, GA and Carolina, PR. (B) Measuring contact angles at different growth stages. Y-axis shows the contact angle in degrees from the surface of a water droplet over

a bacterial lawn. X- axis shows the growth stage or the drying method used. Log, denotes logarithmic phase; Stat, stationary Phase, and drying methods are denoted as "Air dry" and "Silica beads". Error bars represent one standard deviation based on multiple at least three replicates.

### 3.4.3 Optimization of experimental system for CCN activity measurements

To validate our experimental setup, we first tested whether or not the pure water used showed any CCN activity (e.g., due to the organics/solutes) before the cells were introduced. Not surprisingly, pure water produced hundred of small particles (Fig 3.3) that have small level of CCN activity, but could interfere in our analysis. In addition, the supernatant from the last cell pellet wash also contain many particles with CCN activity. The particles present in the water and the supernatant were typically smaller than 100 nm in size, while the bacterial cells were larger than 800 nm. In order to remove these small particles, a pump counterflow virtual impactor (Boulter, Cziczo et al. 2006) was added to the instrumentation pipeline. This device provides a specific cut-off to remove small particles and allow only larger particles go through. Using this devise, particles smaller than 100 nm were reduced from thousands (> 15,000 particles $cm^{-3}$) to a couple of hundred (< 200 particles $cm^{-3}$). However, the high number of small particles in comparison with the number of large particles (e.g., cells) provided inconclusive results. A cut-off smaller than 2 μm to preferentially select large particles was not possible, since the PCVI would have a pressure drop that does not allow the use of the particles or the CCN counters downstream. For that reason, a Diffusional Mobility Analyzer (DMA) was added to the pipeline to select for particles in the size range of interest. Bacterial presence in the pipeline was confirmed by collecting a filter after the DMA and before the CCN counter for microscopy imaging. Fluorescent microscopy showed the present of bacterial cells after the DMA in the size range selected for by the voltage assigned to the

instrument. In addition, Scanning Electron Microscopy (SEM) showed that no or very few abiotic particles were present in the CCN input line (Fig 3.4). Hence, a robust and reliable instrumentation pipeline for studying CCN activity of intact cells was developed and validated.

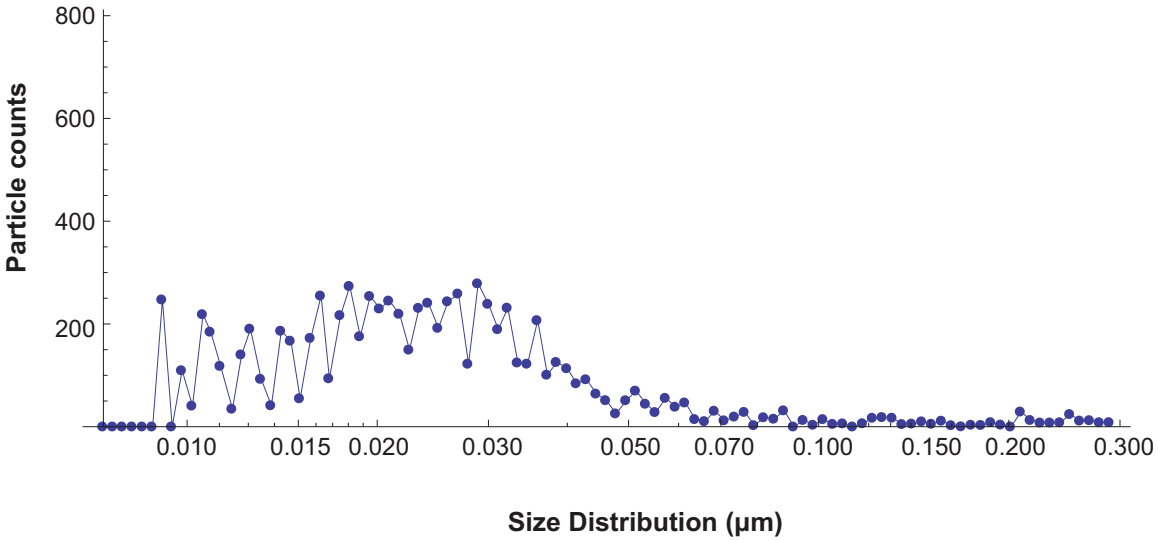


**Figure 3.3 Particle size distribution in blank water sample. Particle** size (x- axis) and particle counts (y-axis) of pure water sample as measured by the Scanning Mobility Particle Sizer (SMPS) during the optimization of the CCN measurement method.

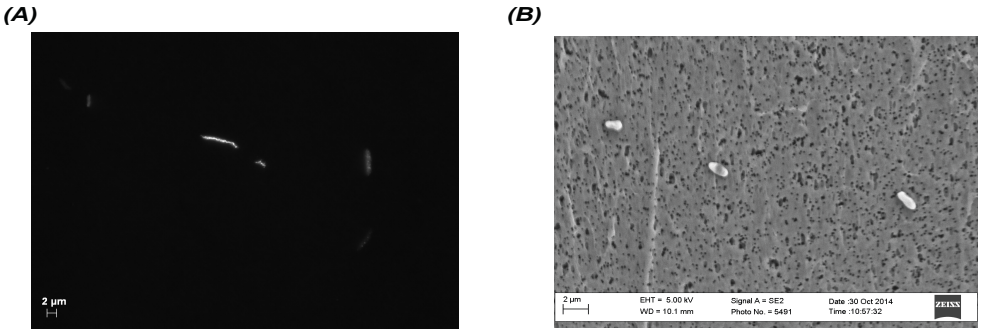


**Figure 3.4 Microscopy images of bacterial cells collected after the DMA.** (**A**) Fluorescent microscope images of samples collected after the DMA confirmed the presence of intact bacterial cell passing through our instrumentation. (**B**) Scanning Electron Microscopy (SEM) analysis confirmed the absence of small abiotic particles in the input to the CCN counter and the presence of intact bacterial cells.

### 3.4.4 CCN activity measurements of bacterial isolates

In total, six isolates were selected to measure their CCN activity, three hydrophilic and three hydrophobic. Cell concentration, measured with the CPC, was in the range of 0.5 – 3 particles $cm^{-3}$. The CCN counter was initially set at 0% supersaturation (~100% relative humidity); particles should not activate at this supersaturation. Consistent with the latter expectation, particles detected at this supersaturation by the CCN counter were of 1 – 3 μm diameter, which was indistinguishable from the size of the bacterial cells selected by the DMA (800 nm to 1000 nm) that entered the CCN counter (water droplets would have been much larger than 1 μm if bacterial cells activated as CCN under the tested supersaturation condition given the size of the cells).

Supersaturation was then increased gradually until 0.6%. After supersaturation increased inside the column, a shift in the droplet size distribution was observed (Fig 3.5 A). Based on the size observed at 0% supersaturation, activation as CCN is determined when the droplet size is larger than 3 μm. Droplet size distribution from hydrophilic bacteria showed a slight shift between 0.1 to 0.15%, where droplet size started increasing with the increase in supersaturation (note peaks after 0.15% supersaturation in Fig 3.5A). In the case of hydrophobic bacteria the shift in droplet size occurred between 0.15 to 0.2% (note the peak after 0.2% supersaturation in Fig. 3.5A). Further, the CCN activation curves can also reveal the CCN (total number of CCN active particles) to CN (total number of particles) ratio for each supersaturation. The critical supersaturation is defined as the saturation at which 50% of the particles are CCN active. Both hydrophobic and

hydrophilic groups reached a plateau in terms of CCN:CN ratio around 0.3% supersaturation; however, hydrophilic bacteria showed a critical supersaturation of 0.1%, while the same value for hydrophobic bacteria was 0.2% (Fig 3.5B). Collectively, the results presented here show that 100% of the cells exposed to increasing supersaturation conditions activated as CCN, and that the hydrophobic organisms showed more fluctuations in the activation ratio compared to their hydrophilic counterparts.

## 3.5 Discussion

Atmospheric models are used to quantify the impact of aerosols in cloud formation. These models require knowledge of the critical supersaturation at which different aerosol types activate as CCN. Classical nucleation theory explains that a particle with a given hygroscopicity and diameter should activate at a specific critical supersaturation (Pruppacher, Klett et al. 1998, Seinfeld and Pandis 2012). A limited number of studies have measured the CCN activity of bacterial cells using different approaches (Franc and DeMott 1998, Bauer, Giebl et al. 2003). Only four different bacteria representative were analyzed during these studies. Their results showed discrepancies in the critical supersaturation, ranging between 0.07 to 1%. Further, these previous studies did not assess the cell constituents or potential physiological adaptations that might be responsible for the CCN activity. In this study, bacterial hygroscopicity and CCN activity was

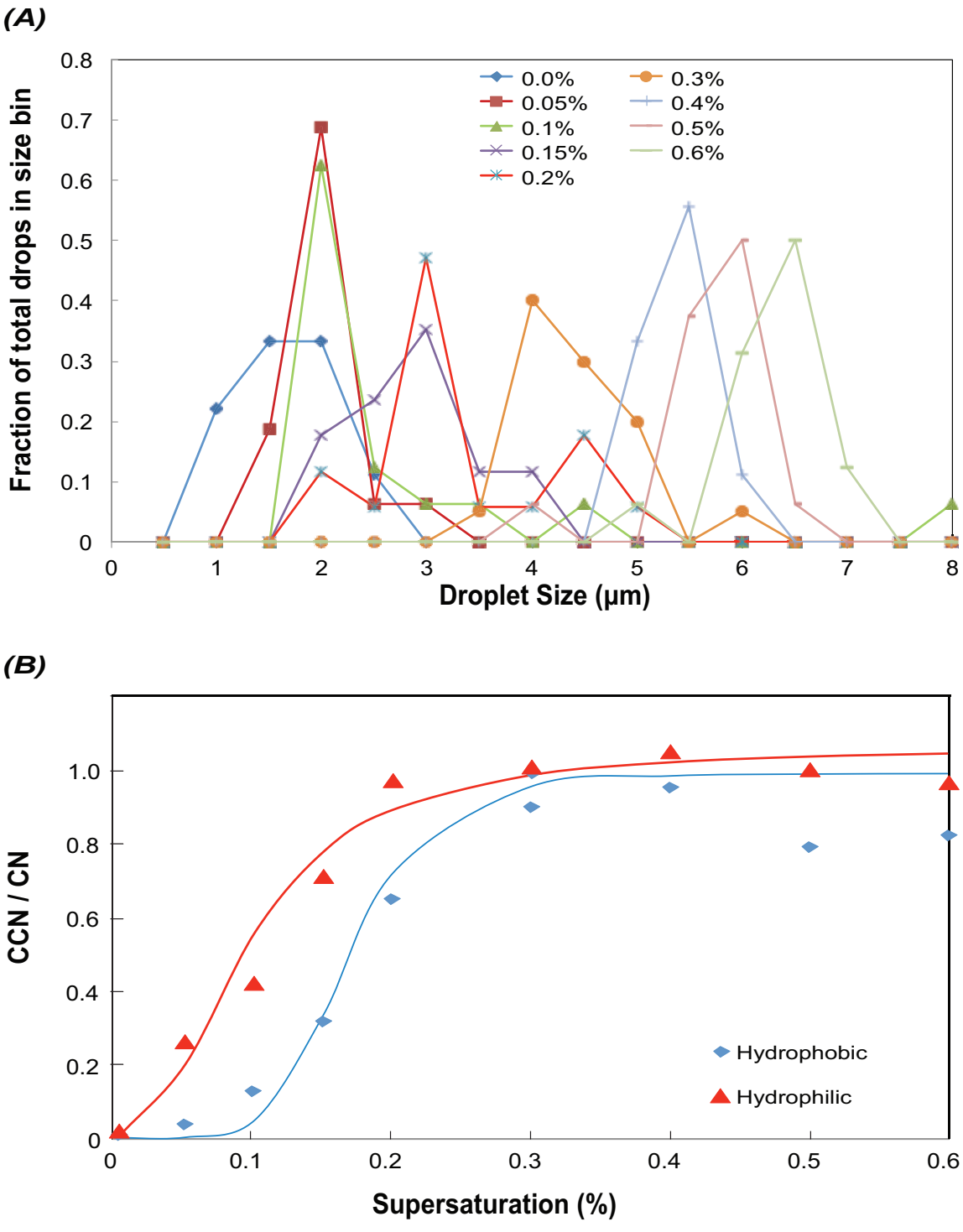


**Figure 3.5 Measuring CCN activity of bacterial isolates with our instrumentation.** (**A**) Example of the droplet size distribution for a hydrophobic isolate at different supersaturation conditions (figure key). Droplet size (x-axis) is measured for each supersaturation during 100 s. (**B**) CCN/CN activation curve for hydrophobic (blue) and hydrophilic (red) isolates. Y- axis shows the CCN to CN ratio and the x-axis the supersaturations conditions (over 100% relative humidity). Data points represents the average of three isolates in each group and the line represents the fit of a sigmoid model to the data obtained.

measured and found to be higher than previously noted or predicted by theory (i.e., classical nucleation theory and global circulation models), even for hydrophobic organisms (Fig 3.4). In particular, even the most hydrophobic organisms tested here showed critical supersaturation 0.3% or less; hydrophilic organisms activated at significantly lower critical supersaturations.

Simulations performed with the atmospheric general circulation model CAM5.0 show that the annual mean maximum supersaturation ($S_{max}$) in liquid clouds is often between 0.1% and 0.2% over continental areas such as the United States, the Amazon, and Asia, indicating that hydrophilic bacteria like those analyzed here could indeed, influence cloud formation and increase precipitation over large regions of the planet. Further, average concentration of bacteria in the atmosphere ranges from $10^{-3}$ to $10^{-2}$ cells $cm^{-3}$ (Harrison, Jones et al. 2005), representing a very low percentage of the total aerosol particles that could activate as CCN (around 1 in 10,000). However, bacterial cells can contribute substantially to the population of gCCN due to their size, which is about 1 – 3 μm in diameter. It has been shown that a concentration as small as $10^{-4}$ – $10^{-2}$ gCCN $cm^{-3}$ is enough to initiate drizzle and precipitation (Feingold, Cotton et al. 1999, Jung, Albrecht et al. 2015). The influence of gCCN in precipitation is found mostly over continental areas where the concentration of small CCN is larger than that over the ocean (Kuba and Takeda 1983, Jung, Albrecht et al. 2015). Given that the concentrations of gCCN and bacterial cells are within an order of magnitude in such areas, our results collectively indicate that airborne bacteria cells are likely an underrepresented pool of particles in the atmosphere and have potential to influence cloud formation and enhance precipitation events.

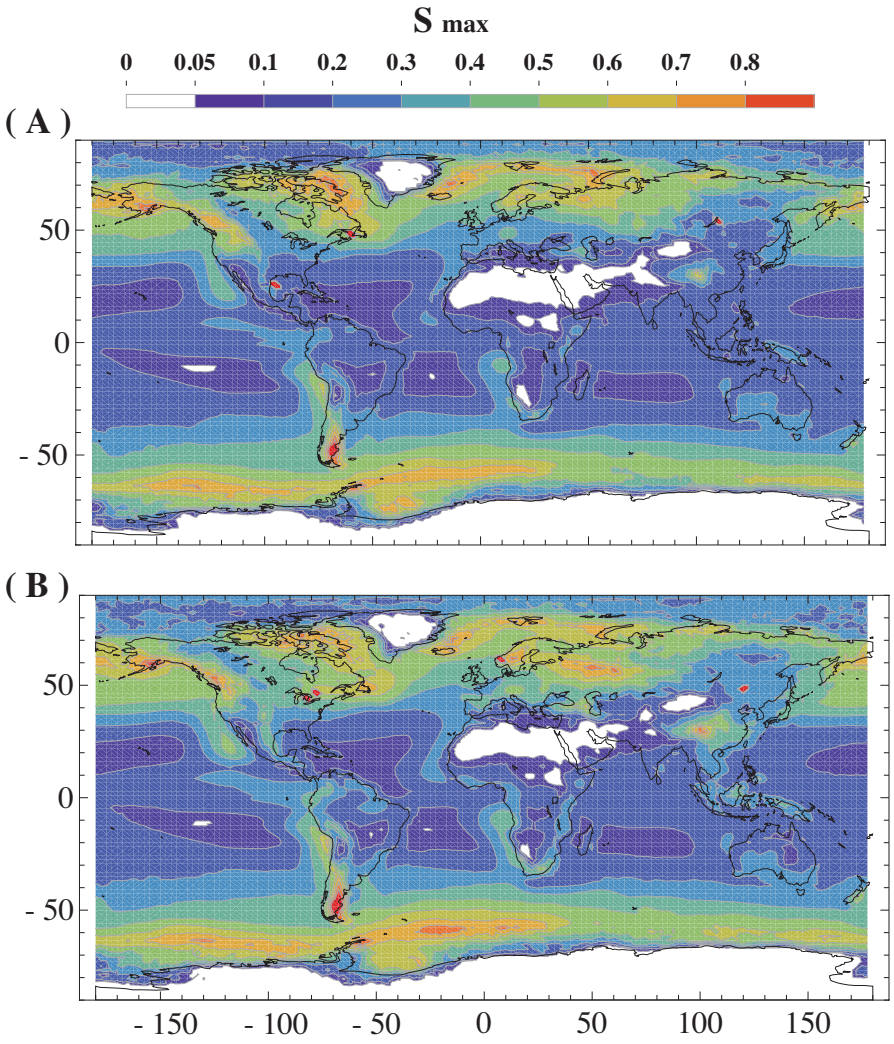


**Figure 3.6 Global plots of in-cloud maximum supersaturation.** The contour plots show the annual averages of the in-cloud supersaturation for the present-day aerosol scenario (A) and for the pre-industrial aerosol emissions scenario (B) corresponding to the 948 hPa pressure level (the third level from the surface), for which the extent of warm cloud cover reaches a peak.

Moreover, several strains of bacteria have been shown to be very effective ice nucleators at high temperatures (e.g., *Pseudomonas syringae* cells freeze at 2°C) acting on the immersion-freezing mode (Vali, Christensen et al. 1976, Möhler, Georgakopoulos et al. 2008, Morris, Sands et al. 2008). This mode requires the presence of an Ice Nuclei (IN) inside a supercooled water droplet in order to initiate freezing. Hence, if a particle serves as an efficient CCN, in addition to IN, it has higher likelihood to condense water

around it and initiate freezing. For this reason, characterizing the ability of bacteria as CCN is crucial for understanding their IN activity (and ice clouds at high altitudes and low ambient temperatures). From all known efficient IN bacterial species, only *Erwinia* sp. were isolated from our samples and showed a critical supersaturation of 0.1%, similar to *Pseudomonas* sp. isolates (Fig 3.2A and Fig 3.4B). Previous measurements of the critical supersaturation for *Erwinia carotovora* was 1% and only 25 – 30 % of the cells activated (Franc and DeMott 1998). The difference between our and the previous results may be due to the different strain of *Erwinia* used and/or differences in the underlying methods used.

Modeling studies have shown that bacterial cells that are not incorporated into clouds have a longer residence time in the atmosphere than those with higher affinity to uptake water and/or being CCN or IN active (Burrows, Butler et al. 2009). Those bacterial strains with longer residence time in the atmosphere could then reach higher altitudes and get transported over longer distances. For example, hydrophobic bacteria such as *Bacillus* sp. are not effective CCN, and these organisms have been detected at altitudes over 10 km and frequently recovered from atmospheric samples (Harrison, Jones et al. 2005, Amato, Parazols et al. 2007, Amato, Parazols et al. 2007, Smith, Griffin et al. 2010). In contrast, hydrophilic bacteria are incorporated into clouds since they are more effective CCN by activating at lower supersaturation, and consequently are scavenged more efficiently from the atmosphere, resulting in shorter residence time. Hence, the results reported here have also implications for the residence time of different bacterial species in the atmosphere.

In order to measure the CCN activity of bacteria using a stream-wise thermal gradient cloud condensation nuclei chamber (Roberts and Nenes 2005), a clean, particle-free system is needed. Small particles associated to water, growth media, and bacterial secretions could interfere with the CCN activation measurements by activating at different, possibly lower, supersaturation than bacterial cells. Several methods that were proposed previously [e.g., Franc and DeMott (1998)] were employed in our study but revealed higher concentrations of small particles compared to those of bacterial cells used in our pipeline, which could affect the CCN counts. Our preliminary assessment showed that these small particles were associated with growth media, fragments of cells and even salts in water. A pump counterflow virtual impactor was not effective enough in removing the small particles due mostly to a pressure drop in the system (Boulter, Cziczo et al. 2006, Kulkarni, Pekour et al. 2011). To overcome this limitation, a DMA was included in the system to select for particles in the size range of bacterial cells, similar to the approach described in Bauer, Giebl et al. (2003). In addition, microscopy imaging was employed to confirm the presence of bacterial cells after the DMA and the absence of small particles in the system, providing a robust and reliable system to measure CCN activity of bacterial cells (Fig 3.1A after part #2).

Different bacteria species can show different degrees of hygroscopicity. For example *P. aeruginosa* has a contact angle of 106° and *P. fluorescens* of 38° (Sharma and Hanumantha Rao 2002) and Van der Mei, Bos et al. (1998) found that even different strains of *S. epidermis* may show a wide range of contact angles (Van der Mei, Bos et al. 1998). This difference could be related to extracellular proteins produced by one of the organisms. In our study, similar variation in contact angles was observed for members of

the *Microbacterium* and *Staphylococcus* genera. Our work also showed no differences in the contact angle of cells harvested at different growth phases but contact angle was substantial affect by the humidity conditions (Fig 3.2B). The latter results may be relevant for *in situ* processes in the atmosphere, where conditions are frequently characterized by low humidity and can change drastically due to weather patterns.

Here, we present a robust and reliable method to measure the CCN activity of intact bacterial cells in the laboratory, producing a clean, particle-free bacterial input stream. Our results showed that the hygroscopicity of bacterial cells could influence their ability to serve as CCN, and potentially participate in cloud formation. These results could be relevant for bacterial transport models as well, since affinity to clouds and water vapors can determine the residence time of cells in the atmosphere. Hydrophilic bacteria have the potential to promote cloud formation over extensive zones of the globe and possibly initiate drizzle in warm clouds formed over the continents. This study also provided a direct link between membrane hygroscopicity and CCN activity of bacterial cells, which is significant given that classical nucleation theory cannot explain the high CCN activity of several bacterial species. The instrumentation pipeline reported here provides also the means to start studying the cell constituents and physiological adaptations that underlie CCN activity and its variation depending on the environmental conditions. The pipeline can also be instrumental in assessing the CCN activity of many more isolates to be recovered from the atmosphere, toward a more quantitative understanding of the importance of bioaerosols for the atmospheric processes.

## 3.6 Acknowledgments

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# CHAPTER 4

# CONCLUSIONS AND RECOMMENDATIONS

## 4.1 Conclusions and Recommendations

In the past decades, bacteria in the atmosphere have become an important topic among biologists and atmospheric scientists because their concentrations at different altitudes might be relevant for atmospheric processes and the hydrological cycle. Previous studies have hypothesized that bacteria can influence the formation of clouds and precipitation (Constantinidou, Hirano et al. 1990, Franc and DeMott 1998, Bauer, Kasper-Giebl et al. 2002, Bauer, Giebl et al. 2003, Amato, Parazols et al. 2007, Christner, Cai et al. 2008, Christner, Morris et al. 2008, Morris, Sands et al. 2008, Burrows, Butler et al. 2009, Burrows, Elbert et al. 2009, Christner 2010, Hoose, Kristjánsson et al. 2010, Morris, Sands et al. 2011, Attard, Yang et al. 2012, Garcia, Hill et al. 2012, DeLeon-Rodriguez, Lathem et al. 2013, Huffman, Prenni et al. 2013, Hill, Moffett et al. 2014, Morris, Conen et al. 2014). The hypothesis is based on the fact that several bacterial species can excrete an outer-membrane protein that promotes ice formation (ice+ bacteria) at temperatures as high as -2°C (Vali, Christensen et al. 1976, Sands, Langhans et al. 1982) and initiate the formation of cloud droplets at low supersaturations (Franc and DeMott 1998, Bauer, Giebl et al. 2003). Beyond this, however, very little quantitative understanding exists on the prevalence of these organisms in the upper atmosphere, their cloud condensation nuclei (CCN) efficiency, and more importantly, which cell properties control the observed CCN activity. The objectives of this study were: i) to quantify and

characterize the bacteria present in the upper troposphere (DeLeon-Rodriguez, Lathem et al. 2013), and ii) to determine whether the affinity of different bacterial cells to water (i.e., degree of hygroscopicity) alone is sufficient to explain cell ability to act as CCN (DeLeon-Rodriguez, Bougiatioti et al, *in preparation*).

Towards closing these gaps in knowledge, we obtained several samples from rainwater (collected on ground) and ambient air in the upper troposphere (collected at altitudes up to 10 km). The majority of the samples were collected on board a specialized aircraft as part of a NASA campaign held in 2010. By analyzing a subset of these samples associated with tropical hurricanes, we have shown (DeLeon-Rodriguez, Lathem et al. 2013) that microbial communities in the atmosphere are complex and over 60% of the cells aloft remain viable. In addition, the concentration of bacterial cells was comparable to that of non-biological IN particles and giant CCN (Kuba and Takeda 1983, Feingold, Cotton et al. 1999, Jung, Albrecht et al. 2015) in these samples. Further, our analysis revealed that bacterial taxa known to be efficient IN and CCN such as plant pathogens of the *Proteobacteria* phylum were absent at high altitudes (8-10 km high). These findings indicated that microorganisms that are efficient CCN or IN contribute to cloud formation and precipitation at lower altitudes during updraft and thus, are scavenged out from the atmosphere. Collectively, these results have challenged several existing paradigms and suggest that the life cycle of bioaerosols may be more relevant to cloud formation and precipitation than previously anticipated.

To obtain a more complete understanding of the role of bacteria in the hydrological cycle we collected over 20 bacterial isolates, representing a phylogenetically diverse group of species, from rainwater and air samples to measure their hygroscopicity and CCN activity. Previous studies hypothesized that cell membrane composition is directly related to CCN activity of bacteria. Contact angle of a water droplet over the bacterial lawn was used to measure the degree of hygroscopicity of these isolates, essentially as performed previously (Weiss, Rosenberg et al. 1982, Van der Mei, Weerkamp et al. 1987, Van der Mei, Bos et al. 1998, Van der Mei, Van de Belt-Gritter et al. 2003), prior to measure CCN activity. The majority of the isolates were found to be hydrophilic, with the exception of four that were hydrophobic. In general, our results showed that exposing the isolates to drier conditions resulted in increased hydrophobicity of the cells. Based on these results, atmospheric conditions could affect the physiology of bacteria, and possibly their CCN activity.

Measuring CCN activity was found to be a challenging task that required extensive testing and optimization. Here we presented a robust and reliable method to measure CCN activity of bacteria cells that is impervious to interference of small abiotic particles such as salts dissolved in water of the culture media. The critical supersaturation that bacteria activated as CCN ranged between 0.1 – 0.2 % and was typically lower for hydrophilic vs. hydrophobic isoaltes (e.g., 0.1 vs. 0.2-0.3% supersaturation). Global atmospheric models showed that a large part of the globe have warm clouds with a maximum supersaturation of 0.2%. Bacterial cells concentration in the atmosphere are similar to those of giant CCN over the continents (Feingold, Cotton et al. 1999, Harrison, Jones et al. 2005, DeLeon-Rodriguez, Lathem et al. 2013, Jung, Albrecht et al. 2015).

Our results collectively indicated that airborne bacterial cells have the potential, in terms of supersaturation and in-situ concentrations, to impact the initiation of drizzle in continental warm clouds and contribute to cloud formation. Hence, bacterial cells may be underrepresented in current atmospheric models. Still, a larger collection of bacterial isolates needs to be analyzed, including their in-situ abundance and dynamics in the atmosphere, in order to have a more complete understanding of the role of bacteria in the hydrological cycle.

This thesis also brought in more sharp focus several questions that should be addressed in the future:

- Based on contact angle, bacteria with hygroscopicity level over 12° will have a critical supersaturation of 5%. However, results showed that critical supersaturation ranged between 0.1 and 0.2%. These results do not agree with the theory, which implies that another property(-ies) of the membrane most likely influences water condensation over the cell (possibly, similar to InaZ for IN activity). Studies are needed to investigate which cell constituents or physical properties are linked to and explained the activity CCN in bacteria.

- Preliminary results showed that isolates Test002 and Test004, identified as *Bacillus* sp., have IN activity similar to homogeneous freezing (-38°C), while *P. syringae* is active at temperatures as high as -2°C (manuscript in preparation). This study observed a difference in activity between hydrophilic and hydrophobic

isolates, but it is unclear to what extent this difference in hydroscopicity also translates to (different) IN activity. Several isolates studied here have the potential to be IN active since they belong to the *Pseudomonas* and *Erwinia* genera that include the most efficient IN bacteria. Hence, IN activity should be tested for all isolates. Based on the preliminary results obtained here and nucleation theory, we hypothesized that hydrophobic bacteria will activate at substantially lower temperatures compared to hydrophilic bacteria.

- Biological IN activity is mediated by an outer-membrane protein encoded by the ice nucleation gene (*inaZ*). The diversity of this gene is limited to *P. syringae*, and a few other culturable bacterial species. The distribution and concentration of the *inaZ* gene in the environment is poorly understood. The quantification of this gene in the environment could provide information of the possible role of bacteria in the formation of ice clouds. Recently, a set of PCR primers was developed for the quantification of the *inaZ* gene present in *P. syringae*. Still, no study has measured the abundance of this key protein in the atmosphere. Quantification of INA positive bacteria may represent important information to include in atmospheric and cloud resolving models.

- The *inaZ* gene has only a few representatives in the databases, due to cultivation biases. Metagenomic analysis could help elucidate the natural diversity of this gene in the atmosphere and other environments. In addition, metagenomic analysis can elucidate the genes that allow bacteria to survive in the atmosphere

for extended periods and have a role in other atmospheric processes, e.g., oxidize organic compounds present in the atmosphere. A limited number of studies have used metagenomic tools to interrogate the genomic diversity in the atmosphere, with the majority of such studies being focused on other environments (e.g., ocean, human, soil). The low biomass present in the atmosphere has represented a challenge in employing genomic analysis of atmospheric samples due to the requirement for relatively high amounts of DNA/protein. However, recent technological advances in sequencing technology will allow the more efficient study of the atmosphere in the near future.

New tools are emerging to help understand the influence of bacteria in atmospheric processes. We were able to obtained 16S rRNA gene amplicons from the upper troposphere (Chapter 2) and measure CCN activity of a variety of bacterial taxa under different growth conditions (Chapter 3). The resulting information may be relevant for cloud-resolving models. Bacterial cells represent a type of aerosol that has not been incorporated in such models, but the concentration of bacteria and their activity in cloud formation as CCN could be more important than previously anticipated. More extensive surveys are important and necessary to obtain a complete understanding of the role of microorganisms in the atmosphere. However, answering the remaining questions in aerobiolgy will require a highly interdisciplinary effort. The join efforts of atmospheric scientists, molecular biologists, and bioinformaticians are necessary to move the field forward.

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**Appendix A: Taxonomic classification of all OTUs recovered in GRIP samples.** The number of sequences of each OTU found in each sample is shown. Letters in the taxonomic affiliation lineage column represent the taxonomic rank as follows: p= phylum, c= class, o= order, f= family, g= genus. Sequences that were not assignable to one of these taxonomic ranks were assigned to the root instead (unassigned sequences; shown in the first row of the table). Asterisks (*) denote the OTUs grouped under 'Others' in Figure 2A.

| Taxonomic affiliation | Aug10 CA Coast | Aug13 CA to FL | Aug17 Clouds | Aug29 Earl | Aug30 Earl | Sep16 Karl Cat1-2 | Sep17 Karl Cat2-3 | Sep17 Low Altitude | Sep20 Few Clouds |
|---|---|---|---|---|---|---|---|---|---|
| * *Root* | 14 | 5 | 96 | 10 | 16 | 9 | 3 | 14 | 16 |
| * *Root;p__ABY1_OD1* | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| * *Root;p__Acidobacteria* | 0 | 5 | 88 | 5 | 3 | 0 | 0 | 0 | 0 |
| * *Root;p__Acidobacteria;c__;o__;f__Koribacteraceae; g__CandidatusKoribacter* | 0 | 0 | 30 | 0 | 6 | 0 | 0 | 0 | 0 |
| *Root;p__Acidobacteria;c__Acidobacteria; o__Acidobacteriales;f__Acidobacteriaceae* | 9 | 0 | 227 | 11 | 9 | 0 | 3 | 0 | 2 |
| * *Root;p__Acidobacteria;c__Chloracidobacteria* | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 |
| * *Root;p__Acidobacteria;c__Holophagae;o__Holophagales;f __Holophagaceae* | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| * *Root;p__Acidobacteria;c__Solibacteres;o__Solibacterales; f__Solibacteraceae;g__CandidatusSolibacter* | 9 | 1 | 30 | 3 | 0 | 1 | 2 | 0 | 2 |
| * *Root;p__Actinobacteria;c__Actinobacteria* | 0 | 0 | 7 | 0 | 0 | 0 | 0 | 0 | 0 |
| * *Root;p__Actinobacteria;c__Actinobacteria;o__0319-7L14* | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 1 |
| * *Root;p__Actinobacteria;c__Actinobacteria; o__Acidimicrobiales* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| * *Root;p__Actinobacteria;c__Actinobacteria; o__Acidimicrobiales;f__CL500-29* | 0 | 0 | 40 | 4 | 3 | 1 | 0 | 0 | 0 |
| * *Root;p__Actinobacteria;c__Actinobacteria; o__Acidimicrobiales;f__Iamiaceae* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| * *Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales* | 11 | 0 | 11 | 7 | 0 | 1 | 2 | 12 | 11 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__ACK-M1* | 5 | 16 | 324 | 14 | 24 | 1 | 7 | 4 | 6 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Actinomycetaceae; g__Actinomyces* | 3 | 0 | 1 | 3 | 0 | 1 | 21 | 0 | 34 |
| ** Root;p__Actinobacteria;c__Actinobacteria ;o__Actinomycetales;f__Actinomycetaceae; g__Varibaculum* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Bogoriellaceae;g__Georgenia* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Cellulomonadaceae; g__Actinotalea* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 9 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Corynebacteriaceae; g__Corynebacterium* | 27 | 0 | 0 | 8 | 22 | 7 | 16 | 6 | 60 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Dermabacteraceae; g__Brachybacterium* | 0 | 0 | 8 | 2 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Dermacoccaceae* | 3 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Dermacoccaceae;g__Kytococcus* | 0 | 3 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Dietziaceae;g__Dietzia* | 0 | 1 | 0 | 1 | 3 | 1 | 0 | 1 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Frankiaceae;g__Frankia* | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Gordoniaceae;g__Gordonia* | 0 | 4 | 1 | 0 | 6 | 1 | 1 | 1 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Intrasporangiaceae* | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Intrasporangiaceae; g__Phycicoccus* | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 2 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Jonesiaceae;g__Jonesia* | 0 | 0 | 1 | 0 | 2 | 0 | 0 | 0 | 0 |
| *Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Microbacteriaceae* | 12 | 8 | 14 | 3 | 7 | 3 | 2 | 5 | 5 |
| ** Root;p__Actinobacteria;c__Actinobacteria;* | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 4 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *o__Actinomycetales;f__Microbacteriaceae;g__Agrococcus* | | | | | | | | | |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Microbacteriaceae; g__CandidatusAquiluna* | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Microbacteriaceae;g__Cryocola* | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Microbacteriaceae; g__Curtobacterium* | 0 | 0 | 0 | 0 | 1 | 7 | 1 | 2 | 5 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Microbacteriaceae; g__Microbacterium* | 11 | 22 | 0 | 1 | 10 | 8 | 8 | 27 | 8 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Micrococcaceae* | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria ;o__Actinomycetales;f__Micrococcaceae;g__Arthrobacter* | 0 | 10 | 0 | 140 | 2 | 0 | 4 | 11 | 3 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Micrococcaceae;g__Kocuria* | 0 | 0 | 0 | 0 | 3 | 4 | 2 | 2 | 4 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Micrococcaceae;g__Micrococcus* | 0 | 0 | 0 | 0 | 2 | 4 | 17 | 4 | 1 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Micrococcaceae;g__Rothia* | 4 | 0 | 0 | 0 | 1 | 6 | 72 | 7 | 77 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Micromonosporaceae* | 6 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Micromonosporaceae; g__Micromonospora* | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Mycobacteriaceae; g__Mycobacterium* | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Nocardiaceae;g__Rhodococcus* | 0 | 41 | 5 | 9 | 7 | 9 | 13 | 25 | 4 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Nocardioidaceae* | 0 | 0 | 0 | 0 | 0 | 9 | 2 | 3 | 7 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Nocardioidaceae; g__Friedmanniella* | 0 | 0 | 0 | 0 | 0 | 5 | 2 | 2 | 2 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Nocardioidaceae;g__Marmoricola* | 0 | 5 | 0 | 3 | 0 | 0 | 0 | 1 | 0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Nocardioidaceae;g__Nocardioides* | 0 | 0 | 0 | 1 | 0 | 7 | 3 | 14 | 5 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Nocardioidaceae;g__Pimelobacter* | 0 | 0 | 6 | 0 | 0 | 1 | 1 | 1 | 5 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Propionibacteriaceae; g__Microlunatus* | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 2 |
| *Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Propionibacteriaceae; g__Propionibacterium* | 140 | 17 | 15 | 19 | 41 | 185 | 39 | 71 | 19 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Sanguibacteraceae* | 0 | 0 | 0 | 0 | 1 | 0 | 4 | 0 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Coriobacteriales;f__Coriobacteriaceae;g__Collinsella* | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Rubrobacterales;f__Rubrobacteraceae; g__Rubrobacter* | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 0 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Solirubrobacterales* | 0 | 4 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| ** Root;p__Actinobacteria;c__Actinobacteria; o__Solirubrobacterales;f__Patulibacteraceae; g__Patulibacter* | 0 | 0 | 0 | 0 | 0 | 2 | 7 | 0 | 0 |
| ** Root;p__AD3;c__JG37-AG-4* | 0 | 0 | 3 | 0 | 1 | 0 | 0 | 0 | 0 |
| ** Root;p__Armatimonadetes;c__Armatimonadia; o__Armatimonadales;f__Armatimonadaceae* | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 |
| ** Root;p__Armatimonadetes;c__CH21* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Bacteroidetes* | 0 | 0 | 15 | 0 | 6 | 0 | 0 | 0 | 1 |
| ** Root;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales* | 0 | 0 | 9 | 0 | 1 | 1 | 0 | 0 | 0 |
| ** Root;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales; f__Porphyromonadaceae* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| ** Root;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales; f__Porphyromonadaceae;g__Porphyromonas* | 0 | 0 | 0 | 0 | 0 | 2 | 24 | 0 | 42 |
| ** Root;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales; f__Prevotellaceae;g__Prevotella* | 4 | 0 | 0 | 49 | 0 | 11 | 0 | 0 | 0 |
| ** Root;p__Bacteroidetes;c__Flavobacteria* | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |

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|---|---|---|---|---|---|---|---|---|---|
| ** Root;p__Bacteroidetes;c__Flavobacteria; o__Flavobacteriales;f__Cryomorphaceae* | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Bacteroidetes;c__Flavobacteria; o__Flavobacteriales;f__Cryomorphaceae;g__Fluviicola* | 0 | 0 | 94 | 10 | 2 | 0 | 0 | 0 | 2 |
| ** Root;p__Bacteroidetes;c__Flavobacteria; o__Flavobacteriales;f__Flavobacteriaceae* | 0 | 0 | 0 | 0 | 5 | 0 | 6 | 0 | 5 |
| ** Root;p__Bacteroidetes;c__Flavobacteria; o__Flavobacteriales;f__Flavobacteriaceae; g__Capnocytophaga* | 0 | 0 | 0 | 0 | 0 | 0 | 12 | 0 | 27 |
| *Root;p__Bacteroidetes;c__Flavobacteria; o__Flavobacteriales;f__Flavobacteriaceae; g__Chryseobacterium* | 74 | 54 | 419 | 261 | 101 | 43 | 99 | 93 | 100 |
| ** Root;p__Bacteroidetes;c__Flavobacteria; o__Flavobacteriales;f__Flavobacteriaceae; g__Flavobacterium* | 0 | 0 | 0 | 0 | 0 | 4 | 1 | 4 | 6 |
| ** Root;p__Bacteroidetes;c__Flavobacteria; o__Flavobacteriales;f__Flavobacteriaceae;g__Gillisia* | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Bacteroidetes;c__Flavobacteria; o__Flavobacteriales;f__Flavobacteriaceae; g__Wautersiella* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |
| ** Root;p__Bacteroidetes;c__Sphingobacteria; o__Sphingobacteriales* | 51 | 37 | 62 | 189 | 337 | 3 | 9 | 1 | 3 |
| ** Root;p__Bacteroidetes;c__Sphingobacteria; o__Sphingobacteriales;f__Flexibacteraceae* | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 3 | 0 |
| ** Root;p__Bacteroidetes;c__Sphingobacteria; o__Sphingobacteriales;f__Flexibacteraceae; g__Cytophaga* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Bacteroidetes;c__Sphingobacteria; o__Sphingobacteriales;f__Flexibacteraceae; g__Dyadobacter* | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 1 | 3 |
| ** Root;p__Bacteroidetes;c__Sphingobacteria; o__Sphingobacteriales;f__Flexibacteraceae; g__Hymenobacter* | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 1 | 1 |
| ** Root;p__Bacteroidetes;c__Sphingobacteria; o__Sphingobacteriales;f__Saprospiraceae* | 0 | 0 | 7 | 0 | 0 | 1 | 0 | 0 | 0 |
| ** Root;p__Bacteroidetes;c__Sphingobacteria; o__Sphingobacteriales;f__Sphingobacteriaceae* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Bacteroidetes;c__Sphingobacteria; o__Sphingobacteriales;f__Sphingobacteriaceae; g__Pedobacter* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 3 |

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|---|---|---|---|---|---|---|---|---|---|
| * *Root;p__Chlorobi;c__Ignavibacteria;o__Ignavibacteriales; f__Ignavibacteriaceae* | 0 | 0 | 16 | 0 | 0 | 0 | 0 | 0 | 0 |
| * *Root;p__Chlorobi;c__SM1B09* | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 1 |
| * *Root;p__Chloroflexi;c__Anaerolineae* | 0 | 0 | 14 | 0 | 1 | 0 | 2 | 0 | 0 |
| * *Root;p__Chloroflexi;c__Anaerolineae;o__A31; f__KNA6-EB22* | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| * *Root;p__Chloroflexi;c__Anaerolineae;o__A4b* | 0 | 0 | 15 | 0 | 0 | 0 | 0 | 0 | 0 |
| * *Root;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales; f__Anaerolinaceae* | 0 | 0 | 1 | 0 | 4 | 0 | 1 | 0 | 0 |
| * *Root;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales; f__Anaerolinaceae;g__WCHB1-05* | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| * *Root;p__Chloroflexi;c__Anaerolineae;o__envOPS12* | 0 | 0 | 2 | 0 | 1 | 0 | 0 | 0 | 0 |
| * *Root;p__Chloroflexi;c__Anaerolineae; o__SJA-101;f__SHA-31* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| * *Root;p__Chloroflexi;c__Anaerolineae;o__SJA-15* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| * *Root;p__Chloroflexi;c__Chloroflexi;o__Roseiflexales* | 0 | 0 | 0 | 3 | 3 | 0 | 0 | 0 | 0 |
| * *Root;p__Chloroflexi;c__Dehalococcoidetes; o__Dehalococcoidales;f__Dehalococcoidaceae* | 0 | 0 | 2 | 0 | 5 | 0 | 0 | 0 | 0 |
| * *Root;p__Chloroflexi;c__Ktedonobacteria* | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 |
| * *Root;p__Chloroflexi;c__SOGA31* | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 4 | 1 |
| * *Root;p__Chloroflexi;c__Thermomicrobia;o__HN1-15* | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 13 | 0 |
| * *Root;p__Cyanobacteria* | 0 | 0 | 6 | 3 | 0 | 0 | 0 | 0 | 0 |
| * *Root;p__Cyanobacteria;c__4C0d-2;o__mle1-12* | 16 | 0 | 0 | 0 | 0 | 1 | 0 | 10 | 3 |
| * *Root;p__Cyanobacteria;c__Chloroplast;o__Stramenopiles* | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |

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|---|---|---|---|---|---|---|---|---|---|
| ** Root;p__Cyanobacteria;c__Chloroplast;o__Streptophyta* | 11 | 0 | 5 | 1 | 5 | 4 | 4 | 5 | 2 |
| ** Root;p__Cyanobacteria;c__Nostocophycideae; o__Nostocales;f__Rivulariaceae;g__Calothrix* | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Cyanobacteria;c__Oscillatoriophycideae* | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| ** Root;p__Cyanobacteria;c__Oscillatoriophycideae; o__Chroococcales;f__Xenococcaceae; g__Chroococcidiopsis* | 0 | 0 | 0 | 0 | 0 | 0 | 7 | 0 | 2 |
| ** Root;p__Cyanobacteria;c__Synechococcophycideae; o__Synechococcales;f__Synechococcaceae; g__Prochlorococcus* | 26 | 0 | 72 | 5 | 0 | 1 | 0 | 0 | 7 |
| ** Root;p__Elusimicrobia;c__Elusimicrobia; o__Elusimicrobiales* | 0 | 0 | 0 | 0 | 0 | 3 | 1 | 14 | 0 |
| ** Root;p__Firmicutes* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| ** Root;p__Firmicutes;c__Bacilli;o__Bacillales* | 0 | 0 | 0 | 0 | 0 | 5 | 1 | 0 | 1 |
| ** Root;p__Firmicutes;c__Bacilli;o__Bacillales; f__Alicyclobacillaceae;g__Alicyclobacillus* | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 |
| ** Root;p__Firmicutes;c__Bacilli;o__Bacillales; f__Bacillaceae;g__Anoxybacillus* | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Bacilli;o__Bacillales; f__Bacillaceae;g__Bacillus* | 13 | 1 | 0 | 22 | 6 | 8 | 4 | 1 | 4 |
| ** Root;p__Firmicutes;c__Bacilli;o__Bacillales; f__Bacillaceae;g__Geobacillus* | 0 | 2 | 2 | 0 | 0 | 4 | 2 | 0 | 2 |
| ** Root;p__Firmicutes;c__Bacilli;o__Bacillales; f__Listeriaceae;g__Listeria* | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Bacilli;o__Bacillales; f__Paenibacillaceae;g__Ammoniphilus* | 0 | 0 | 7 | 0 | 0 | 1 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Bacilli;o__Bacillales; f__Paenibacillaceae;g__Brevibacillus* | 0 | 1 | 0 | 0 | 0 | 3 | 0 | 5 | 0 |
| ** Root;p__Firmicutes;c__Bacilli;o__Bacillales; f__Paenibacillaceae;g__Paenibacillus* | 8 | 5 | 2 | 0 | 0 | 0 | 1 | 0 | 0 |
| ** Root;p__Firmicutes;c__Bacilli;o__Bacillales; f__Planococcaceae* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Bacilli;o__Bacillales; f__Planococcaceae;g__Planococcus* | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 5 | 0 |
| ** Root;p__Firmicutes;c__Bacilli;o__Bacillales; f__Staphylococcaceae;g__Staphylococcus* | 20 | 4 | 0 | 14 | 8 | 10 | 9 | 26 | 5 |

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|---|---|---|---|---|---|---|---|---|---|
| ** Root;p__Firmicutes;c__Bacilli;o__Exiguobacterales; f__Exiguobacteraceae;g__Exiguobacterium* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| ** Root;p__Firmicutes;c__Bacilli;o__Gemellales; f__Gemellaceae;g__Gemella* | 0 | 0 | 0 | 2 | 0 | 14 | 107 | 6 | 65 |
| ** Root;p__Firmicutes;c__Bacilli;o__Lactobacillales; f__Aerococcaceae;g__Abiotrophia* | 0 | 0 | 0 | 1 | 0 | 12 | 28 | 1 | 22 |
| ** Root;p__Firmicutes;c__Bacilli;o__Lactobacillales; f__Carnobacteriaceae;g__Granulicatella* | 4 | 11 | 0 | 0 | 1 | 0 | 14 | 4 | 1 |
| ** Root;p__Firmicutes;c__Bacilli;o__Lactobacillales; f__Carnobacteriaceae;g__Marinilactibacillus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| ** Root;p__Firmicutes;c__Bacilli;o__Lactobacillales; f__Enterococcaceae;g__Enterococcus* | 7 | 7 | 62 | 3 | 1 | 3 | 0 | 1 | 1 |
| ** Root;p__Firmicutes;c__Bacilli;o__Lactobacillales; f__Lactobacillaceae;g__Lactobacillus* | 6 | 0 | 0 | 1 | 0 | 6 | 0 | 0 | 1 |
| ** Root;p__Firmicutes;c__Bacilli;o__Lactobacillales; f__Leuconostocaceae;g__Leuconostoc* | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Bacilli;o__Lactobacillales; f__Streptococcaceae* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| ** Root;p__Firmicutes;c__Bacilli;o__Lactobacillales; f__Streptococcaceae;g__Lactococcus* | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 0 |
| *Root;p__Firmicutes;c__Bacilli;o__Lactobacillales; f__Streptococcaceae;g__Streptococcus* | 19 | 4 | 0 | 12 | 28 | 93 | 555 | 32 | 453 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales* | 2 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Clostridiaceae* | 0 | 0 | 6 | 0 | 8 | 0 | 0 | 0 | 0 |
| *Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Clostridiaceae;g__Clostridium* | 16 | 7 | 281 | 15 | 19 | 1 | 6 | 5 | 3 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__ClostridialesFamilyXI.IncertaeSedis* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__ClostridialesFamilyXI.IncertaeSedis;g__Anaerococcus* | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 2 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__ClostridialesFamilyXI.IncertaeSedis;g__Finegoldia* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__ClostridialesFamilyXI.IncertaeSedis;g__Peptoniphilus* | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__ClostridialesFamilyXI.IncertaeSedis; g__Sedimentibacter* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |

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|---|---|---|---|---|---|---|---|---|---|
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__ClostridialesFamilyXIII.IncertaeSedis* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Dehalobacteriaceae;g__Dehalobacterium* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Lachnospiraceae* | 0 | 8 | 3 | 2 | 0 | 0 | 0 | 0 | 2 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Lachnospiraceae;g__Blautia* | 0 | 0 | 0 | 1 | 0 | 0 | 2 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Lachnospiraceae;g__Coprococcus* | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Lachnospiraceae;g__Eubacterium* | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Lachnospiraceae;g__Oribacterium* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Lachnospiraceae;g__Roseburia* | 0 | 0 | 0 | 8 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Peptococcaceae;g__Dehalobacter* | 2 | 0 | 0 | 1 | 0 | 1 | 2 | 4 | 3 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Ruminococcaceae* | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Ruminococcaceae;g__Faecalibacterium* | 0 | 0 | 0 | 19 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Ruminococcaceae;g__Oscillospira* | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Symbiobacteriaceae* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__ThermoanaerobacteralesFamilyIII.IncertaeSedis; g__Thermoanaerobacterium* | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Veillonellaceae* | 0 | 0 | 14 | 3 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Veillonellaceae;g__Desulfosporomusa* | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Veillonellaceae;g__Dialister* | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Veillonellaceae;g__Mitsuokella* | 0 | 0 | 0 | 18 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Firmicutes;c__Clostridia;o__Clostridiales; f__Veillonellaceae;g__Veillonella* | 0 | 0 | 3 | 1 | 0 | 0 | 10 | 0 | 2 |

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|---|---|---|---|---|---|---|---|---|---|
| ** Root;p__Fusobacteria;c__Fusobacteria; o__Fusobacteriales;f__Fusobacteriaceae; g__Fusobacterium* | 0 | 0 | 0 | 0 | 0 | 5 | 8 | 0 | 30 |
| ** Root;p__Fusobacteria;c__Fusobacteria; o__Fusobacteriales;f__Fusobacteriaceae;g__Leptotrichia* | 0 | 0 | 0 | 0 | 0 | 13 | 18 | 3 | 81 |
| ** Root;p__Gemmatimonadetes;c__Gemmatimonadetes* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Gemmatimonadetes;c__Gemmatimonadetes; o__Gemmatimonadales;f__Gemmatimonadaceae; g__Gemmatimonas* | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__GOUTA4;c__RB384* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Nitrospirae;c__Nitrospira;o__Nitrospirales; f__Nitrospiraceae;g__Nitrospira* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| ** Root;p__Nitrospirae;c__Nitrospira;o__Nitrospirales; f__Thermodesulfovibrionaceae;g__DCE29* | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__OP3;c__koll11;o__GIF10;f__kpj58rc* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Planctomycetes;c__Phycisphaerae* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Planctomycetes;c__Phycisphaerae; o__Phycisphaerales* | 0 | 0 | 3 | 2 | 0 | 0 | 2 | 0 | 0 |
| ** Root;p__Planctomycetes;c__Planctomycea; o__Gemmatales;f__Gemmataceae* | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Planctomycetes;c__Planctomycea; o__Gemmatales;f__Gemmataceae;g__Gemmata* | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Planctomycetes;c__Planctomycea; o__Gemmatales;f__Isosphaeraceae* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| ** Root;p__Planctomycetes;c__Planctomycea; o__Pirellulales* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| ** Root;p__Planctomycetes;c__Planctomycea; o__Pirellulales;f__;g__Rhodopirellula* | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Planctomycetes;c__Planctomycea; o__Pirellulales;f__Pirellulaceae* | 0 | 2 | 11 | 1 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Planctomycetes;c__Planctomycea; o__Pirellulales;f__Pirellulaceae;g__A17* | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 |
| ** Root;p__Planctomycetes;c__vadinHA49* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria* | 0 | 0 | 25 | 3 | 0 | 2 | 3 | 4 | 3 |

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|---|---|---|---|---|---|---|---|---|---|
| ** Root;p__Proteobacteria;c__Alphaproteobacteria* | 0 | 15 | 34 | 0 | 5 | 4 | 2 | 0 | 0 |
| *Root;p__Proteobacteria;c__Alphaproteobacteria; o__Caulobacterales;f__Caulobacteraceae* | 429 | 270 | 89 | 266 | 564 | 7 | 13 | 15 | 3 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Caulobacterales;f__Caulobacteraceae; g__Brevundimonas* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 3 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales* | 6 | 0 | 21 | 5 | 2 | 4 | 6 | 10 | 7 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__;g__Nordella* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Aurantimonadaceae;g__Aurantimonas* | 0 | 6 | 0 | 0 | 0 | 2 | 0 | 1 | 3 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Beijerinckiaceae* | 0 | 0 | 4 | 0 | 3 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Bradyrhizobiaceae* | 140 | 69 | 19 | 23 | 20 | 2 | 0 | 8 | 2 |
| *Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Bradyrhizobiaceae;g__Afipia* | 3958 | 2372 | 885 | 320 | 536 | 16 | 52 | 79 | 28 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Bradyrhizobiaceae;g__Balneimonas* | 0 | 3 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Bradyrhizobiaceae;g__Bosea* | 1 | 15 | 5 | 4 | 5 | 2 | 2 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Bradyrhizobiaceae; g__Rhodopseudomonas* | 2 | 4 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Brucellaceae;g__Pseudochrobactrum* | 0 | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Hyphomicrobiaceae;g__Devosia* | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 9 | 10 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Hyphomicrobiaceae; g__Hyphomicrobium* | 159 | 39 | 65 | 6 | 1 | 2 | 0 | 1 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Hyphomicrobiaceae; g__Pedomicrobium* | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Hyphomicrobiaceae; g__Rhodomicrobium* | 7 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |

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|---|---|---|---|---|---|---|---|---|---|
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Hyphomicrobiaceae;g__Rhodoplanes* | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 |
| *Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Methylobacteriaceae; g__Methylobacterium* | 247 | 24 | 17 | 3 | 6 | 20 | 12 | 41 | 10 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Phyllobacteriaceae;g__Mesorhizobium* | 0 | 0 | 0 | 3 | 19 | 0 | 2 | 20 | 2 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Rhizobiaceae;g__Agrobacterium* | 14 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Rhizobiaceae;g__Kaistia* | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 4 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Rhizobiaceae;g__Rhizobium* | 3 | 0 | 0 | 0 | 0 | 1 | 3 | 0 | 2 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Rhodobiaceae* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Xanthobacteraceae;g__Labrys* | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhodobacterales;f__Hyphomonadaceae* | 0 | 0 | 7 | 0 | 0 | 0 | 0 | 1 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhodobacterales;f__Rhodobacteraceae* | 0 | 0 | 0 | 3 | 0 | 3 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhodobacterales;f__Rhodobacteraceae; g__Paracoccus* | 0 | 0 | 0 | 0 | 0 | 1 | 3 | 2 | 10 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhodobacterales;f__Rhodobacteraceae; g__Rubellimicrobium* | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 8 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhodospirillales;f__Acetobacteraceae* | 4 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhodospirillales;f__Acetobacteraceae;g__Acidocella* | 0 | 0 | 0 | 0 | 1 | 31 | 29 | 80 | 47 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhodospirillales;f__Acetobacteraceae; g__Roseomonas* | 0 | 0 | 9 | 0 | 0 | 0 | 2 | 0 | 0 |
| *Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhodospirillales;f__Rhodospirillaceae* | 134 | 36 | 29 | 130 | 236 | 13 | 1 | 8 | 5 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rhodospirillales;f__Rhodospirillaceae;g__Skermanella* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria;* | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 1 |

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|---|---|---|---|---|---|---|---|---|---|
| *o__Rickettsiales* | | | | | | | | | |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rickettsiales;f__;g__CandidatusOdyssella* | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rickettsiales;f__;g__CandidatusPelagibacter* | 5 | 0 | 117 | 10 | 0 | 1 | 0 | 6 | 2 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Rickettsiales;f__Rickettsiaceae* | 0 | 0 | 8 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Sphingomonadales* | 1 | 4 | 0 | 0 | 2 | 10 | 7 | 9 | 4 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Sphingomonadales;f__Erythrobacteraceae* | 0 | 0 | 0 | 0 | 0 | 1 | 5 | 0 | 1 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Sphingomonadales;f__Erythrobacteraceae; g__Erythrobacter* | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Sphingomonadales;f__Erythrobacteraceae; g__Lutibacterium* | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 6 | 7 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Sphingomonadales;f__Sphingomonadaceae* | 39 | 3 | 1 | 1 | 0 | 5 | 17 | 1 | 7 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Sphingomonadales;f__Sphingomonadaceae; g__Kaistobacter* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Sphingomonadales;f__Sphingomonadaceae; g__Novosphingobium* | 0 | 11 | 13 | 2 | 3 | 8 | 0 | 0 | 2 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Sphingomonadales;f__Sphingomonadaceae; g__Sphingobium* | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Root;p__Proteobacteria;c__Alphaproteobacteria; o__Sphingomonadales;f__Sphingomonadaceae; g__Sphingomonas* | 151 | 36 | 39 | 6 | 18 | 1 | 8 | 12 | 5 |
| ** Root;p__Proteobacteria;c__Alphaproteobacteria; o__Sphingomonadales;f__Sphingomonadaceae; g__Sphingopyxis* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria* | 0 | 0 | 18 | 0 | 0 | 1 | 4 | 25 | 1 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales* | 1 | 0 | 8 | 2 | 2 | 3 | 2 | 12 | 10 |

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|---|---|---|---|---|---|---|---|---|---|
| *Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__;g__Aquabacterium* | 413 | 368 | 168 | 168 | 360 | 7 | 15 | 3 | 4 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__;g__Methylibium* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 3 | 0 |
| *Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__;g__Mitsuaria* | 0 | 2 | 1 | 1388 | 1 | 0 | 2 | 0 | 0 |
| *Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__;g__Paucibacter* | 153 | 310 | 73 | 170 | 281 | 4 | 24 | 37 | 22 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__;g__Roseateles* | 0 | 0 | 0 | 1 | 0 | 0 | 2 | 0 | 1 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Alcaligenaceae* | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Alcaligenaceae;g__Achromobacter* | 0 | 2 | 2 | 0 | 0 | 14 | 18 | 39 | 32 |
| *Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Burkholderiaceae;g__Burkholderia* | 0 | 0 | 0 | 1 | 0 | 165 | 148 | 381 | 207 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Burkholderiaceae;g__Cupriavidus* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Burkholderiaceae; g__Polynucleobacter* | 10 | 0 | 15 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Burkholderiaceae;g__Ralstonia* | 11 | 11 | 0 | 10 | 1 | 2284 | 1954 | 4868 | 2797 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Comamonadaceae* | 0 | 0 | 42 | 5 | 2 | 67 | 32 | 98 | 84 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Comamonadaceae;g__Acidovorax* | 30 | 8 | 0 | 0 | 0 | 2 | 3 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Comamonadaceae; g__Comamonas* | 0 | 0 | 0 | 0 | 2 | 4 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Comamonadaceae;g__Curvibacter* | 0 | 0 | 0 | 0 | 0 | 72 | 62 | 153 | 63 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Comamonadaceae;g__Delftia* | 0 | 0 | 0 | 0 | 0 | 3 | 1 | 7 | 0 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Comamonadaceae; g__Diaphorobacter* | 7 | 27 | 0 | 6 | 7 | 1 | 11 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Comamonadaceae;* | 0 | 1 | 14 | 0 | 3 | 2 | 0 | 0 | 0 |

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|---|---|---|---|---|---|---|---|---|---|
| *g__Limnohabitans* | | | | | | | | | |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Comamonadaceae;g__Rhodoferax* | 0 | 0 | 16 | 3 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Comamonadaceae;g__Variovorax* | 0 | 0 | 0 | 0 | 0 | 6 | 4 | 8 | 3 |
| *Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Oxalobacteraceae* | 8 | 10 | 23 | 7 | 12 | 447 | 373 | 867 | 533 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Oxalobacteraceae;g__Collimonas* | 0 | 0 | 11 | 0 | 1 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Oxalobacteraceae; g__Janthinobacterium* | 0 | 1 | 1 | 1 | 1 | 7 | 3 | 1 | 2 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Oxalobacteraceae;g__Massilia* | 0 | 21 | 0 | 4 | 0 | 5 | 1 | 7 | 2 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Methylophilales;f__Methylophilaceae* | 10 | 4 | 33 | 3 | 3 | 0 | 2 | 0 | 1 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Methylophilales;f__Methylophilaceae; g__Methylophilus* | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 2 | 0 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Neisseriales;f__Neisseriaceae* | 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Neisseriales;f__Neisseriaceae;g__Aquitalea* | 0 | 1 | 0 | 2 | 2 | 4 | 7 | 18 | 14 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Neisseriales;f__Neisseriaceae;g__Eikenella* | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 0 | 3 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Neisseriales;f__Neisseriaceae;g__Neisseria* | 0 | 3 | 0 | 1 | 0 | 7 | 64 | 0 | 46 |
| ** Root;p__Proteobacteria;c__Betaproteobacteria; o__Rhodocyclales* | 2 | 0 | 5 | 0 | 1 | 0 | 0 | 7 | 2 |
| ** Root;p__Proteobacteria;c__Deltaproteobacteria* | 0 | 0 | 8 | 0 | 3 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Deltaproteobacteria; o__Bdellovibrionales;f__Bdellovibrionaceae; g__Bdellovibrio* | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Deltaproteobacteria; o__Desulfobacterales;f__Desulfobulbaceae* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| ** Root;p__Proteobacteria;c__Deltaproteobacteria;o__MIZ46* | 0 | 0 | 6 | 0 | 2 | 0 | 0 | 2 | 0 |

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|---|---|---|---|---|---|---|---|---|---|
| ** Root;p__Proteobacteria;c__Deltaproteobacteria; o__Myxococcales* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Deltaproteobacteria; o__Myxococcales;f__Cystobacteraceae* | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Deltaproteobacteria; o__Syntrophobacterales;f__Syntrophaceae* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Deltaproteobacteria; o__Syntrophobacterales;f__Syntrophaceae; g__Syntrophus* | 0 | 0 | 12 | 0 | 3 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Deltaproteobacteria; o__Syntrophobacterales;f__Syntrophobacteraceae; g__Syntrophobacter* | 2 | 0 | 9 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Epsilonproteobacteria; o__Campylobacterales;f__Campylobacteraceae; g__Arcobacter* | 11 | 0 | 7 | 0 | 0 | 0 | 0 | 0 | 1 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria* | 0 | 0 | 6 | 0 | 0 | 4 | 6 | 7 | 3 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Aeromonadales;f__Aeromonadaceae;g__Aeromonas* | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Root;p__Proteobacteria;c__Gammaproteobacteria; o__Alteromonadales;f__Shewanellaceae;g__Shewanella* | 20 | 7 | 133 | 8 | 12 | 2 | 5 | 1 | 6 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Cardiobacteriales;f__Cardiobacteriaceae; g__Cardiobacterium* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Chromatiales* | 0 | 0 | 0 | 0 | 0 | 5 | 6 | 41 | 6 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Chromatiales;f__Sinobacteraceae* | 131 | 106 | 55 | 4 | 9 | 0 | 1 | 2 | 0 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Enterobacteriales;f__Enterobacteriaceae* | 1 | 0 | 9 | 2 | 2 | 8 | 0 | 3 | 6 |
| *Root;p__Proteobacteria;c__Gammaproteobacteria; o__Enterobacteriales;f__Enterobacteriaceae; g__Escherichia* | 0 | 0 | 124 | 45 | 939 | 0 | 0 | 1 | 0 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Enterobacteriales;f__Enterobacteriaceae;g__Serratia* | 0 | 2 | 0 | 0 | 0 | 2 | 1 | 1 | 2 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Legionellales* | 0 | 0 | 7 | 0 | 0 | 2 | 0 | 1 | 0 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Legionellales;f__Legionellaceae* | 0 | 0 | 5 | 0 | 0 | 3 | 0 | 0 | 0 |

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|---|---|---|---|---|---|---|---|---|---|
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Legionellales;f__Legionellaceae;g__Legionella* | 0 | 6 | 1 | 0 | 0 | 2 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Oceanospirillales;f__FCPT525* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Oceanospirillales;f__Halomonadaceae;g__Halomonas* | 0 | 5 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Oceanospirillaceae;g__Marinomonas* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Pasteurellales;f__Pasteurellaceae;g__Haemophilus* | 0 | 0 | 0 | 0 | 0 | 0 | 15 | 0 | 16 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Pseudomonadales;f__Moraxellaceae* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Pseudomonadales;f__Moraxellaceae; g__Acinetobacter* | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Pseudomonadales;f__Pseudomonadaceae* | 0 | 0 | 1 | 0 | 3 | 3 | 2 | 0 | 1 |
| *Root;p__Proteobacteria;c__Gammaproteobacteria; o__Pseudomonadales;f__Pseudomonadaceae; g__Pseudomonas* | 1 | 4 | 10 | 5 | 3 | 6 | 13 | 21 | 20 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Thiotrichales* | 0 | 0 | 0 | 0 | 0 | 1 | 3 | 2 | 0 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Xanthomonadales;f__Xanthomonadaceae; g__Frateuria* | 0 | 0 | 0 | 0 | 0 | 5 | 3 | 0 | 1 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Xanthomonadales;f__Xanthomonadaceae; g__Luteibacter* | 0 | 7 | 0 | 1 | 1 | 6 | 15 | 22 | 5 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Xanthomonadales;f__Xanthomonadaceae; g__Rhodanobacter* | 0 | 21 | 0 | 2 | 2 | 2 | 6 | 19 | 9 |
| ** Root;p__Proteobacteria;c__Gammaproteobacteria; o__Xanthomonadales;f__Xanthomonadaceae; g__Stenotrophomonas* | 0 | 0 | 0 | 2 | 1 | 2 | 7 | 7 | 5 |
| ** Root;p__Spirochaetes;c__Spirochaetes; o__Spirochaetales;f__Spirochaetaceae;g__SA-8* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 |
| ** Root;p__Tenericutes* | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |

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|---|---|---|---|---|---|---|---|---|---|
| ** Root;p__Tenericutes;c__Erysipelotrichi; o__Erysipelotrichales;f__Erysipelotrichaceae* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Tenericutes;c__Erysipelotrichi; o__Erysipelotrichales;f__Erysipelotrichaceae; g__Clostridium* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Tenericutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae;g__PSB-M-3* | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| ** Root;p__TG3;c__TG3-1;o__GN09* | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Thermi;c__Deinococci* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Thermi;c__Deinococci;o__Deinococcales; f__Deinococcaceae;g__Deinococcus* | 0 | 7 | 0 | 0 | 2 | 0 | 0 | 1 | 0 |
| ** Root;p__TM6;c__SJA-4* | 0 | 0 | 4 | 0 | 1 | 0 | 0 | 0 | 0 |
| ** Root;p__TM7;c__TM7-1* | 0 | 0 | 9 | 0 | 3 | 0 | 0 | 13 | 1 |
| ** Root;p__TM7;c__TM7-3* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| ** Root;p__TM7;c__TM7-3;o__Blgi18* | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 |
| ** Root;p__TM7;c__TM7-3;o__EW055* | 0 | 18 | 8 | 2 | 4 | 8 | 12 | 8 | 16 |
| ** Root;p__Verrucomicrobia* | 0 | 0 | 6 | 0 | 1 | 0 | 0 | 0 | 0 |
| ** Root;p__Verrucomicrobia;c__Methylacidiphilae; o__Methylacidiphilales;f__LD19* | 0 | 0 | 27 | 1 | 2 | 0 | 0 | 0 | 0 |
| ** Root;p__Verrucomicrobia;c__Opitutae;o__Opitutales; f__Opitutaceae* | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Verrucomicrobia;c__Opitutae;o__Opitutales; f__Opitutaceae;g__Opitutus* | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Verrucomicrobia;c__Opitutae; o__Puniceicoccales;f__Puniceicoccaceae* | 0 | 4 | 34 | 4 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Verrucomicrobia;c__Spartobacteria; o__Spartobacteriales;f__Spartobacteriaceae* | 0 | 0 | 11 | 0 | 0 | 0 | 0 | 0 | 0 |
| ** Root;p__Verrucomicrobia;c__Spartobacteria; o__Spartobacteriales;f__Spartobacteriaceae; g__CandidatusXiphinematobacter* | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |

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|---|---|---|---|---|---|---|---|---|---|
| ** Root;p__Verrucomicrobia;c__Spartobacteria; o__Spartobacteriales;f__Spartobacteriaceae; g__Chthoniobacter* | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 0 |
| ** Root;p__Verrucomicrobia;c__Verrucomicrobiae; o__Verrucomicrobiales* | 0 | 0 | 16 | 0 | 3 | 0 | 0 | 0 | 0 |
| ** Root;p__ZB2* | 0 | 0 | 4 | 2 | 0 | 0 | 2 | 3 | 0 |